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# GROWTH

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*Growth*, 1960, **24**, 333-338.

# OOGENESIS IN ADULT *Drosophila melanogaster*. X. STUDIES ON THE BEHAVIOR OF THE FOLLICLE CELLS[1]

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Received June 2, 1960

## INTRODUCTION

The data to be reported represent an extension of work (King, Rubinson and Smith, 1956; King, 1958) in which the process of oogenesis in the adult fruit fly, *Drosophila melanogaster*, was described and the development of the egg subdivided into a series of consecutive stages ending with stage 14, the mature, ovarian, primary oocyte. The time required for the egg to pass through each developmental stage has been estimated and the relative rates of growth of the various components of the developing chamber have been calculated (King, 1957). The developing egg of *Drosophila* consists of a nest of 16 daughter cells which arise from four consecutive divisions of an oogonium. Once the cyst is enveloped by mesodermal follicle cells it pinches off from the germarium and so becomes the first egg chamber (stage 2). Fifteen of the cells differentiate into nurse cells and nourish the most posterior, daughter germ cell which becomes the oocyte. Intercommunication of the cytoplasm between all the members of the 16 cell cyst is made possible by large pores in the walls separating adjacent cells (King and Devine, 1958). At first all 16 germ cells grow at identical rates. However, during vitellogenesis (stages 8 through 11) the oocyte grows at a rate 10 times more rapidly than previously and at the expense of the nurse cells which shrink and eventually degenerate. It is also during these stages that the vitelline membrane is secreted in the space between the follicle cells and the oocyte (King, 1960). Subsequent to stage 11 no further volume increase occurs in the oocyte. The follicle cells secrete the endochorion during stages 12 and 13 and the exochorion during stage 14. This work describes the division, migration and differentiation of

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normal follicle cells as a function of developmental stage and abnormalities characteristic of the follicle cells in two mutants.

## Materials and Methods

Ovaries were studied as Feulgen-stained whole mounts (using the methods of King, Rubinson and Smith, 1956) or as stained sections (using the methods of King, 1960). Developing egg chambers are either spherical or ellipsoidal, and their volume and surface areas therefore could be calculated readily once their dimensions were determined. The following formulas were used in these calculations: volume of a sphere $= 4.189r^3$; surface area of a sphere $= 12.57r^2$; volume of an ellipsoid $= 0.5236w^2l$ (w = width; $l$ = length); and surface area of an ellipsoid $= 6.283ab\left(\frac{\sin^{-1}e}{e} + \sqrt{1\text{-}e^2}\right)$; where $e = \sqrt{1 - \frac{b^2}{a^2}}$ and $a = 0.5l$ and $b = 0.5w$. In determining $\sin^{-1}e$ Chamber's 6-Figure Mathematical Tables by L. J. Comrie, vol. 2 (W. & R. Chambers, Ltd., Edinburgh, 1949) were used (Table VIA. pg. 350, Inverse circular and hyperbolic functions). The number of follicle cells per unit area ($64\mu^2$) of egg chamber surface was determined using a net ocular micrometer while viewing the specimen with an oil immersion objective in a Leitz Ortholux microscope. Given the nuclear density and the chamber surface area the total number of follicle cells per chamber was calculated assuming that each cell was uninucleate. No multinucleate follicle cells have been observed.

## Results and Conclusions

The data on follicle cell number as a function of increasing chamber volume are presented in figure 1. Here volumes are corrected for shrinkage (see King, 1958). The data were first graphed linearly, and the straight lines of best fit calculated for the steeply ascending portion and the gradually descending portion. This graph turned out to be so unwieldy that the lines of best fit were transferred to double log paper where they appear slightly curved. Subsequently the observed values (some 109 points) were added to the graph.

The stage 2 chamber is surrounded by an envelope of approximately 80 cuboidal follicle cells, each about $2\mu$ high. The average

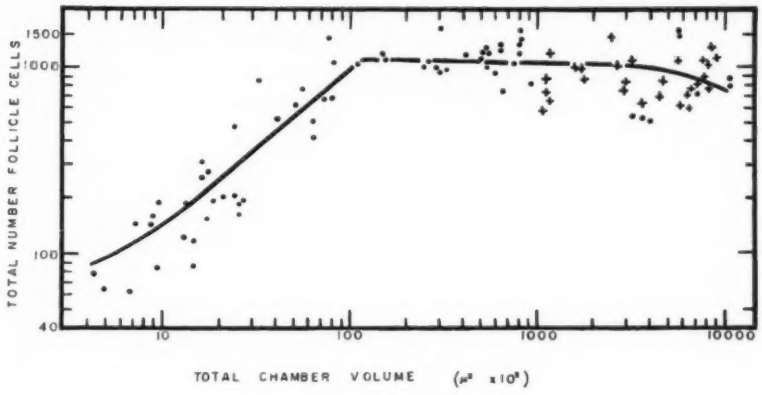


FIGURE 1

The relation between the logarithm of the number of follicle cells and the logarithm of the volume of the developing egg chamber. Dots represent values for chambers covered by a cuboidal epithelium; plus signs represent values for chambers enveloped anteriorly by a squamous and posteriorly by a columnar epithelium.

nuclear density is $4/64\mu^2$. During the next 30 hours the follicle cells divide as the chamber increases in volume. By stage 6 mitosis ceases in the follicular epithelium. The chamber now has a volume of $1 \times 10^5\mu^3$ and is surrounded by about 1,100 cuboidal follicle cells. The average nuclear density is $7/64\mu^2$, and each cell is about $3\mu$ high. By the end of stage 7 the total number of follicle cells is about what it was 20 hours earlier at stage 6. However, the cells have grown, since the average nuclear density is $2/64\mu^2$. The follicle cell nuclei are homogeneously distributed over the chamber. During stage 8 which lasts 4 hours a posteriorward migration of follicle cells is thought to occur which results in an observed gradient of nuclear densities (see figure 2). The follicle cells above the nurse cells show a fairly constant height (averaging $4\mu$). Those above the oocyte, however, show a gradient of heights with the most posterior cells being the tallest ($14\mu$). By stage 10B all the follicle cells above the oocyte have reached or exceeded this height (a maximum height of $25\mu$ has been observed); whereas the follicle cells above the nurse cells have become exceedingly thin (50m$\mu$ according to King and Devine, 1958). The nuclear density of these squamous cells is only 1/15th that of the columnar follicle cells (see figure 3). During vitellogenesis the

nuclear densities above the oocyte drop precipitously from 6 to $0.3/64\mu^2$, and consequently the area covered by each cell increases 20 fold. The period of maximum follicle cell growth is during stages 9 through 12. During this time interval (roughly 1 hour) the average

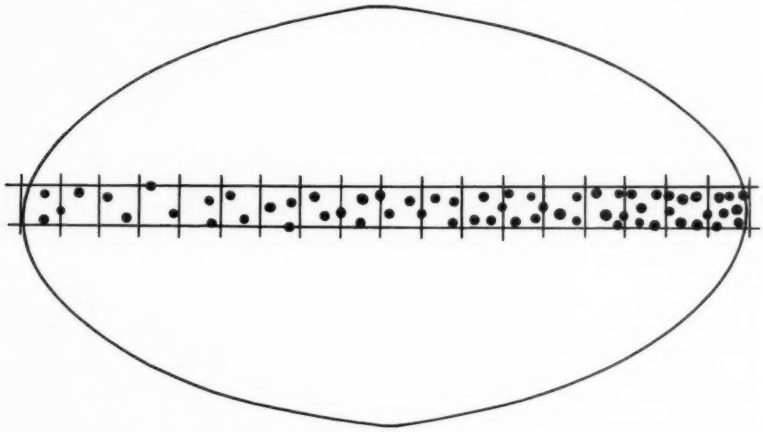

FIGURE 2

Nuclear densities for a series of 18 areas of $64\,\mu^2$ extending across a stage 8 chamber. The anterior and posterior poles are to the left and right, respectively. The dimensions are $88 \times 144\,\mu$.

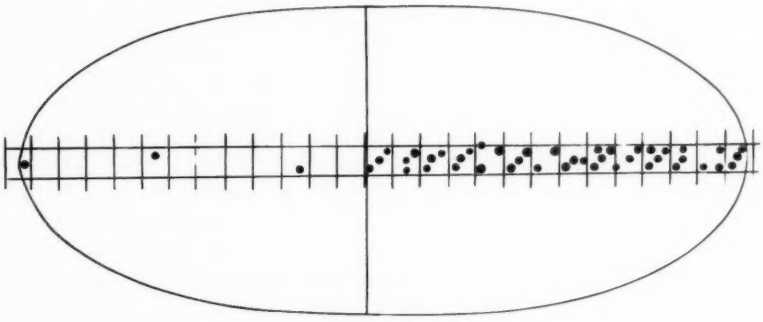

FIGURE 3

Nuclear densities for a series of 27 areas of $64\,\mu^2$ extending across a stage 10A chamber. The anterior and posterior poles are to the left and right, respectively. The dimensions are $90 \times 210\,\mu$.

follicle cell increases in volume between 5 and 10 times. During stages 3-7 nuclear volumes between 5 and $10\mu^3$ have been observed. A marked nuclear growth occurs subsequently. The follicle cells enveloping oocytes at stages 8, 10 and 12 have volumes of 25, 60 and $190\mu^3$, respectively. During stages 6-14 (a time interval of 24 hours under optimal conditions) a gradual decline in follicle cell number from 1,100 to 800 occurs, and the maximum oocyte volume is reached ($\sim 1 \times 10^7\mu^3$). It appears that columnar cells die more often than squamous cells, since they make up 90% of the total number of follicle cells during stages 9 and 10, but 80% during stages 11 and 12. Subsequently the columnar follicle cells decrease in height as the endochorion is formed.

## The Follicle Cells of *ty* and *dm*

The behavior of the follicle cells is distinctly abnormal in females homozygous for either of two, X-chromosomal, female sterile mutations. These mutants are *diminutive* (*dm*) located at 4.6 and *tiny* (*ty*) located at 44.5. Both mutants are characterized by marked retardations of vitellogenesis (see King and Burnett, 1957). Females which are hemizygous for *dm* ($dm/N^{55l}$) show more extreme ovarian abnormalities which suggests that the *dm* gene is hypomorphic rather than amorphic (see Hillman, 1958, for a description of $N^{55l}$).

In the case of late pre-yolk chambers of *ty* females the follicular epithelium is often double layered above the oocyte. In sections the follicular epithelium is seen to contain many degenerating nuclei, and numerous granules which stain with the periodic acid-Schiff procedure are found in the cytoplasm of follicle cells. Some of the anterior follicle cells make a shortened posteriorward migration over the surface of the stage 8 chamber with the result that nuclear density over the posterior two thirds is greater than that above the anterior third. The follicle cells in the area of high nuclear density differentiate into columnar cells; whereas the rest stay cuboidal. The result is that the layer of columnar cells extends abnormally far forward, enveloping about one half of the nurse chamber. The vitelline membrane is thicker than normal. The fact that the follicular epithelium surrounding the nurse chamber is abnormally thick may hinder passage of nutrients from hemolymph to nurse cytoplasm and be the cause of the retarded vitellogenesis of this mutant. It appears that the

absence of the + allele of *ty* becomes critical during the migration and differentiation of follicle cells during stages 7 and 8.

In the case of *dm* the situation is quite different. The surface migration appears to proceed in the usual fashion but the cells normally destined to form a columnar epithelium during stage 9 never increase in height and soon degenerate. This degeneration reminds one of that seen in pyridoxin-deficient ovaries (Sang and King, 1959). Normally the anterior follicle cells surrounding the oocyte migrate centripetally between the oocyte and adjacent nurse cells during stage 10, and the vitelline membrane is extended inward concurrently. This centripetal migration does not occur in the case of *dm*. Degenerating nurse cell nuclei often enter the ooplasm. Border cells are not observed at the appropriate stages (see King, 1960 for a description of these cells). Thus the absence of the + allele of *dm* becomes critical at a later stage than $+^{ty}$. The migration and differentiation of those follicle cells covering the oocyte is controlled by the + allele of *dm*.

## Summary

This work describes the division, migration and differentiation of the follicle cells as a function of developmental stage during oogenesis in *Drosophila melanogaster* and the abnormalities in the behavior of follicle cells seen in two female sterile mutants.

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# SIZE AND SHAPE VARIATION IN THE PAINTED TURTLE.[1] A PRINCIPAL COMPONENT ANALYSIS

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## INTRODUCTION

The concepts of size and shape are fundamental to the analysis of variation in living organisms. And yet, as noted by Simpson, Roe and Lewontin (1960), there is at present no general agreement on practical definitions of size and shape. The adoption of a generally applicable method of measuring size and shape variation would be particularly timely as so much effort is being devoted to the study of morphological and physiological variation within and between plant and animal populations (Anderson, 1954; Prosser, 1955).

Parting biometrical variation into size and shape components is often highly desirable as the size of most organisms is more affected than their shape by fluctuations of the external environment; size variation is also more likely to reflect heterogeneity of samples with respect to age-composition. Shape tends thus generally to provide more reliable indications than size on the internal constitution of organisms; this makes the analysis of size and shape a basic step in the study of biometrical variation.

The present study of the Painted Turtle (*Chrysemys picta marginata*) is an attempt to evaluate the applicability of principal component analysis to size and shape variation in groups of living organisms. The beginnings of principal component analysis are probably to be found in the works of Karl Pearson (1901). The statistical properties of principal components were investigated in detail by

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Hotelling in 1933. Anderson (1958) has given the most comprehensive recent exposition. Principal component and related forms of multivariate analysis have been used by a number of authors (Blackith, 1960; Teissier, 1955; Wright, 1954) in attempts to describe complex growth patterns in terms of a minimum number of basic trends. Recent biometrical studies of character-complexes (Bailey, 1956; Kraus and Choi, 1958; Olson and Miller, 1958) have made increasingly clear that, whenever the discovery of genetical, developmental and functional relationships is contemplated, joint consideration of all characters becomes indispensable and multivariate statistical techniques are indicated. However, the relationships of principal components with the concepts of size and shape do not appear to have been ever examined in detail. This is the main purpose of the present note. A comparative analysis of sex dimorphism in several species of turtles will be presented later.

Among the vertebrates, turtles are perhaps those that are most readily suitable to studies of variation in overall size and shape. Due to a rigid bony carapace, consistent measurements of body dimensions can be obtained. Further, interesting changes of proportions occur in the shell during growth (Mosimann, 1958). Sexual dimorphism in body size and proportions is common. Finally, for aquatic species like the one studied here, shape is probably of major adaptive significance because of the importance of streamlining for locomotion in a dense fluid medium.

## Material and Data

Eighty-four specimens of Midland Painted Turtles (*Chrysemys picta marginata*) were collected in a single day (August 2, 1956) from a small stagnant pond of the St. Lawrence Valley, at Coteau Landing, 35 miles southwest of Montreal, Canada. This material can therefore safely be assumed to represent a single local population. Specimens are preserved in the Department of Biology of the University of Montreal. Only individuals for which the sex was externally discernible are included in the present analysis, one abnormal male being excluded. Carapace dimensions were measured in three mutually perpendicular directions of space: length, maximum width, and height. More detailed definitions of these measurements have been given before (Mosimann, 1958). Thus each specimen is represented in this study by a set of three measurements

(length, width, height)

hereafter designated

$$\mathbf{X} = (X_1, X_2, X_3)$$

for convenience. Measurements rounded to the nearest millimeter are listed in Table 1. Several facts are explicit from examination of the data. (1) In general, long individuals are also wide and high, and inversely, short individuals are narrow and low. Clearly this reflects the fact that length, width and height are influenced by one general factor of variation, size. (2) However, some individuals of the same length show different widths or heights and these differences of proportions constitute shape variation. (3) In addition it can be seen that females attain a larger size than males and also (4) that they tend to be higher relative to length.

TABLE 1
CARAPACE DIMENSIONS OF PAINTED TURTLES (*Chrysemys picta marginata*) IN MM.

| 24 Males | | | 24 Females | | |
|---|---|---|---|---|---|
| length | width | height | length | width | height |
| 93 | 74 | 37 | 98 | 81 | 38 |
| 94 | 78 | 35 | 103 | 84 | 38 |
| 96 | 80 | 35 | 103 | 86 | 42 |
| 101 | 84 | 39 | 105 | 86 | 40 |
| 102 | 85 | 38 | 109 | 88 | 44 |
| 103 | 81 | 37 | 123 | 92 | 50 |
| 104 | 83 | 39 | 123 | 95 | 46 |
| 106 | 83 | 39 | 133 | 99 | 51 |
| 107 | 82 | 38 | 133 | 102 | 51 |
| 112 | 89 | 40 | 133 | 102 | 51 |
| 113 | 88 | 40 | 134 | 100 | 48 |
| 114 | 86 | 40 | 136 | 102 | 49 |
| 116 | 90 | 43 | 137 | 98 | 51 |
| 117 | 90 | 41 | 138 | 99 | 51 |
| 117 | 91 | 41 | 141 | 105 | 53 |
| 119 | 93 | 41 | 147 | 108 | 57 |
| 120 | 89 | 40 | 149 | 107 | 55 |
| 120 | 93 | 44 | 153 | 107 | 56 |
| 121 | 95 | 42 | 155 | 115 | 63 |
| 125 | 93 | 45 | 155 | 117 | 60 |
| 127 | 96 | 45 | 158 | 115 | 62 |
| 128 | 95 | 45 | 159 | 118 | 63 |
| 131 | 95 | 46 | 162 | 124 | 61 |
| 135 | 106 | 47 | 177 | 132 | 67 |

## BIVARIATE ANALYSIS

Whereas the preceding points can be detected by inspection of the data, they are brought out more clearly by plotting pairs of measure-

ments of the various individuals as dots in cartesian coordinate systems. In the resulting bivariate scatter diagrams (Figures 1 and 2), the facts noted above are now graphically evident: (1) dots are spread along the diagonal of each diagram according to size differences and (2) scattered about it because of shape variation. (3) In both diagrams females extend further to the right than males since they grow larger and (4), in the first diagram, they tend to be above males of equal length because of greater relative height.

These phenomena have been made considerably more obvious by outlining the clusters of dots with ninety-five per cent equal frequency ellipses. The equation of the latter can be written in matrix notation as:

$$(\mathbf{X} - \overline{\mathbf{X}})\ \mathbf{W}^{-1}\ (\mathbf{X} - \overline{\mathbf{X}})' = c^2$$

where $\mathbf{X} = (X_1, X_2)$, the pair of coordinates of the locus of the ellipse; $\mathbf{X}' = \begin{bmatrix} X^1 \\ X^2 \end{bmatrix}$, the column-vector obtained by transposing $\mathbf{X}$; $\overline{\mathbf{X}} = (\overline{X}_1, \overline{X}_2)$, the pair of coordinates of the center of the ellipse and the row-vector of sample means; $\mathbf{W} = \begin{bmatrix} W_{11} & W_{12} \\ W_{21} & W_{22} \end{bmatrix}$, the inverse of the matrix of the equation of the ellipse and the covariance matrix of the sample. The value of $c^2$ is equal to that of chi-square with two degrees of freedom (Anderson, 1958: 54), which is 5.99 at the 95 per cent level. The elements of the bivariate mean vectors and covariance matrices for Figures 1 and 2 are all included in the trivariate statistics (Table 2).

To determine an equal frequency ellipse, the direction cosines $\mathbf{U} = \begin{bmatrix} U_{11} & U_{12} \\ U_{21} & U_{22} \end{bmatrix}$ of its principal axes and the diagonal form $\Lambda = \begin{bmatrix} \lambda_1 & 0 \\ 0 & \lambda_2 \end{bmatrix}$ of its covariance matrix are calculated, the subscripts in $\mathbf{U}$ and $\Lambda$ always being ordered by convention so that $\lambda_1 > \lambda_2$. In this study, the elements of matrix $\mathbf{U} = \begin{bmatrix} \cos\theta & \sin\theta \\ -\sin\theta & \cos\theta \end{bmatrix}$ were obtained from 5-place trigonometric tables after evaluating angle $\theta$ from $\tan 2\theta = \dfrac{2\ W_{12}}{W_{11} - W_{22}}$ and $\Lambda$ was then calculated as $\Lambda = \mathbf{UWU}'$, where

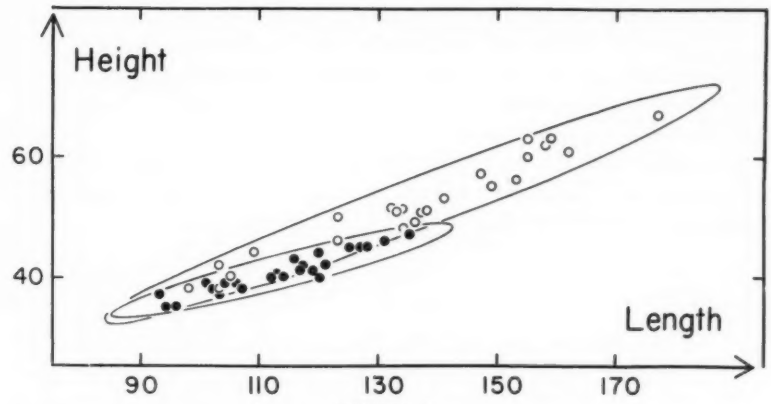


FIGURE 1
Bivariate scatter diagram of carapace height and length. Males, solid circles; females, hollow circles.

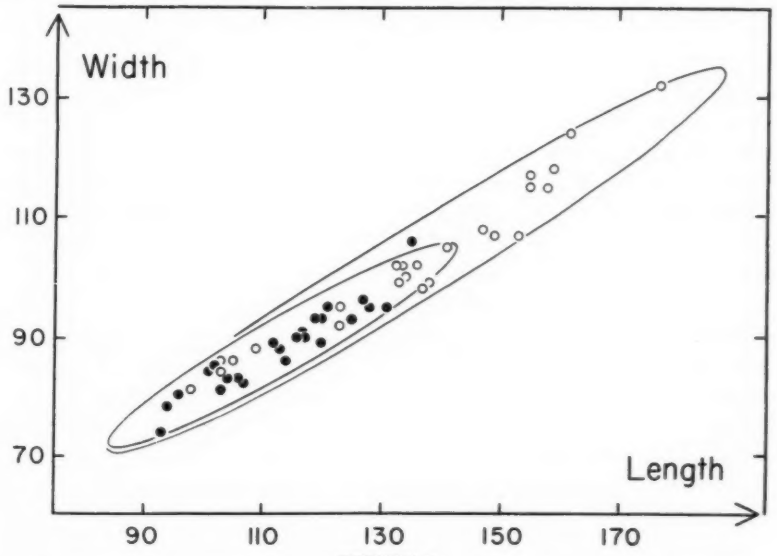


FIGURE 2
Bivariate scatter diagram of carapace width and length. Males, solid circles; females, hollow circles.

U′ is the transpose of matrix U. The principal components of the covariance matrix W are the variances $\lambda_1$ and $\lambda_2$ of the observations along the principal axes of their equal frequency ellipses, the direction cosines of these axes being $(U_{11}, U_{12})$ for the first and $(U_{21}, U_{22})$ for the second.

Providing the coordinate axes are graduated in the same scale, the extremities of the principal axes of a 95 per cent equal frequency ellipse are determined by the following vector equations:

$$\text{major axis} \quad (X_1, X_2) = (\bar{X}_1, \bar{X}_2) \pm \sqrt{5.99\lambda_1}\,(U_{11}, U_{12})$$
$$\text{minor axis} \quad (X_1, X_2) = (\bar{X}_1, \bar{X}_2) \pm \sqrt{5.99\lambda_2}\,(U_{21}, U_{22}).$$

The above equations lay bare the analogy of bivariate with univariate confidence intervals. After these extremities have been plotted, the ellipse can be graphed either by means of an ellipsograph (such as the one made by Riefler) or following a standard drafting procedure.

Were the parameter mean vector and covariance matrix known, and were the distribution of our observations exactly bivariate normal, then equal frequency ellipses would be true confidence intervals. Although this is not the case, our ellipses are estimates of true confidence intervals. Moreover, their main raison d'être in the present study is their descriptive validity, and the latter is confirmed by visual comparison with the clusters of dots of the data. On the other hand, with small samples, the effect of extreme observations is considerable and equal frequency ellipses should be used with caution. Their biometrical utilization has been thoroughly discussed by Defrise-Gussenhoven (1955).

## Trivariate Analysis

While bivariate scatter diagrams with equal frequency ellipses have brought out size and shape differences rather satisfactorily, the present problem is basically trivariate. The well-justified popularity of bivariate techniques stems no doubt largely from the relative simplicity of bivariate computations as well as from the ease of preparation of bivariate scatter diagrams. Graphing is considerably more difficult in three than in two dimensions, and it becomes geometrically impossible when more than three variables are analyzed jointly. Algebraically and numerically, however, multivariate statistical techniques remain identical in pattern beyond the bivariate level.

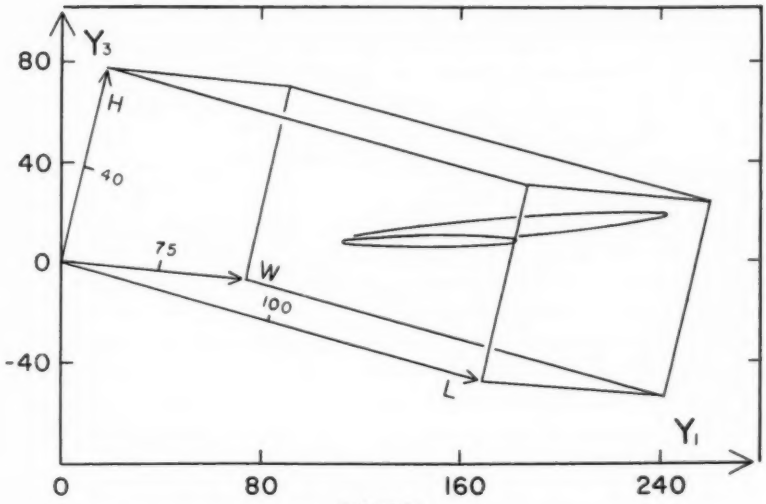


FIGURE 3

The trivariate scatter diagram of carapace length, width and height viewed perpendicularly to the first and third principal axes of males.

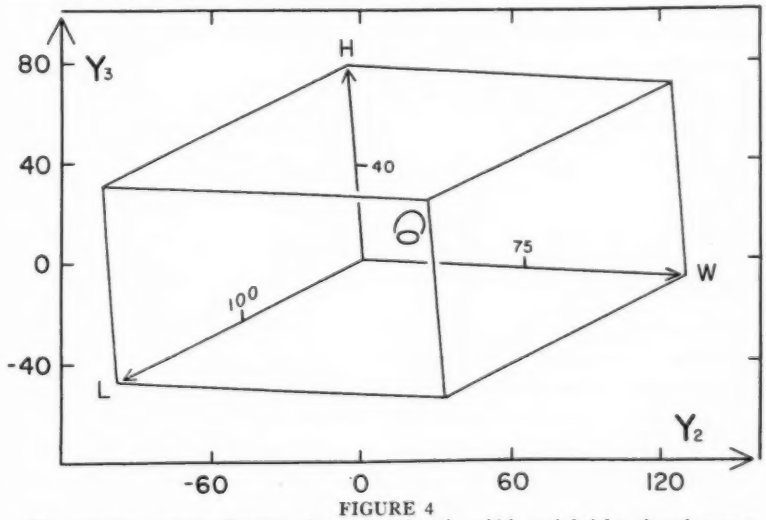


FIGURE 4

The trivariate scatter diagram of carapace length, width and height viewed perpendicularly to the second and third principal axes of males.

Inasmuch as the frequency distribution of observations is approximately multivariate normal, a sample can be summarized by its mean vector $\overline{X}$ and covariance matrix W. In the trivariate case,

$$\overline{X} = (\overline{X}_1, \overline{X}_2, \overline{X}_3) \text{ and } W = \begin{bmatrix} W_{11} & W_{12} & W_{13} \\ W_{21} & W_{22} & W_{23} \\ W_{31} & W_{32} & W_{33} \end{bmatrix}$$

The trivariate statistics relative to (length, width, height) in the present study are listed in Table 2. The elements of the mean vector of females are greater than those of males, reflecting the greater size of the former sex. The elements of covariance matrices are also greater in females, which reflects a greater range of size variation.

Earlier in this paper the analysis was restricted to that of the marginal distributions of the variables two by two. All components of $\overline{X}$ and W were perforce ignored at any one time except those corresponding to two of the variables. Thus the components of $\overline{X}$ other than $(\overline{X}_1, \overline{X}_3)$ and those of W other than $\begin{bmatrix} W_{11} & W_{13} \\ W_{31} & W_{33} \end{bmatrix}$ were automatically discarded in the bivariate analysis of $(X_1, X_3)$. In other words, carapace length and height were analyzed as if width had not been measured, etc. Multivariate techniques alone can take all variables into account jointly and thus provide a unified analytical approach.

TABLE 2

MEAN VECTORS $\overline{X}$ AND COVARIANCE MATRICES W

| | 24 Males | | | 24 Females | | |
|---|---|---|---|---|---|---|
| | length | width | height | length | width | height |
| X | (113.38 | 88.29 | 40.71) | (136.00 | 102.58 | 51.96) |
| W | 138.77 | 79.15 | 37.38 | 451.39 | 271.17 | 168.70 |
| | 79.15 | 50.04 | 21.65 | 271.17 | 171.73 | 103.29 |
| | 37.38 | 21.65 | 11.26 | 168.70 | 103.29 | 66.65 |

With the present trivariate data, a joint scatter diagram of all variables is fortunately still possible. Slanted views of the tridimensional scatter diagram have been calculated and illustrated (Figures 3 and 4), the clusters of male and female dots being represented by ellipsoids.

Analogously with bivariate principal components, multivariate principal components are the variances of observations along the principal

axes of multidimensional equal frequency ellipsoids. In the present case, with three variables, the latter are tridimensional ellipsoids. The evaluation of the direction cosines $U = \begin{bmatrix} U_{11} & U_{12} & U_{13} \\ U_{21} & U_{22} & U_{23} \\ U_{31} & U_{32} & U_{33} \end{bmatrix}$ of the principal axes and of the corresponding covariance matrix $\Lambda = \begin{bmatrix} \lambda_1 & 0 & 0 \\ 0 & \lambda_2 & 0 \\ 0 & 0 & \lambda_3 \end{bmatrix}$ is an eigenvalue and eigenvector problem. Its best general solution is probably the Jacobi method of matrix diagonalization (White, 1958: 395), a generalization of the procedure already described for bivariate covariance matrices. The results of principal component analysis of our data are given in Table 3.

TABLE 3
COVARIANCE MATRICES Λ AND MATRICES OF DIRECTION COSINES U OF THE PRINCIPAL AXES

| | 24 Males | | | 24 Females | | |
|---|---|---|---|---|---|---|
| Λ | 195.28 | 0.00 | 0.00 | 680.40 | 0.00 | 0.00 |
| | 0.00 | 3.69 | 0.00 | 0.00 | 6.50 | 0.00 |
| | 0.00 | 0.00 | 1.10 | 0.00 | 0.00 | 2.86 |
| U | .84012 | .49190 | .22854 | .81263 | .49549 | .30676 |
| | —.48811 | .86938 | —.07696 | —.54537 | .83213 | .10062 |
| | —.23654 | —.04690 | .97049 | —.20340 | —.24907 | .94645 |

## TYPES OF VARIATION CORRESPONDING TO THE PRINCIPAL COMPONENTS

The types of variation occurring in the directions spanned by the principal axes can best be judged from the equations of the latter. Thus the equation of the first principal axis of males is

$$\frac{X_1 - \overline{X}_1}{U_{11}} = \frac{X_2 - \overline{X}_2}{U_{12}} = \frac{X_3 - \overline{X}_3}{U_{13}} = Y_1 - \overline{Y}_1$$

or

$$\frac{X_1 - 113.38}{.84012} = \frac{X_2 - 88.29}{.49190} = \frac{X_3 - 40.71}{.22854} = Y_1 - 147.99$$

upon substitution of the numerical values given in Table 3. The value of $Y_1$ is obtained by rotation of X as explained later. In fact, for a given set $X = (X_1, X_2, X_3)$, the value of $Y_1$ is equal to that of the linear compound $U_{11}X_1 + U_{12}X_2 + U_{13}X_3$. It is noteworthy that the direction cosines of the $Y_1$-axis are all positive. Because of this, the

equation of the major axis corresponds to a simultaneous increase (or decrease) of all variables. For instance, a positive deviation of 100 units in $Y_1$ corresponds approximately to deviations of (+84, +49, +23) millimeters in ($X_1$, $X_2$, $X_3$), that is in carapace (length, width, height). For this reason, and since growth is generally defined as the increase in size of an organism, the first principal component is interpreted as a growth trend. The use of a linear compound like $Y_1 = U_{11}X_1 + U_{12}X_2 + U_{13}X_3$ as a size measure should be very practical in many types of studies, although it calls for careful distinction from the usual volumetric or ponderal definition of size. Size was for instance defined as a volume estimate from a non-linear compound ($Y_1 = k\ X_1\ X_2\ X_3$) and compared with direct volume measurements by Mosimann (1958).

A feature of special significance is the difference in relative magnitude of the direction cosines of male and female major axes, particularly those corresponding to carapace height. $U_{13}$ equals .22854 and .30676 in males and females respectively. A positive deviation of 100 units in size thus corresponds approximately to a deviation in height of +31 mm. in females, but only to +23 mm. in males. This expresses the lower relative growth rate of carapace height in males, a phenomenon noticeable in Figure 1 and brought out previously by regression analysis (Mosimann, 1958).

The second principal axis of males has the equation

$$\frac{X_1 - \overline{X}_1}{U_{21}} = \frac{X_2 - \overline{X}_2}{U_{22}} = \frac{X_3 - \overline{X}_3}{U_{23}} = Y_2 - \overline{Y}_2$$

or $$\frac{X_1 - 113.38}{-.48811} = \frac{X_2 - 88.29}{.86938} = \frac{X_3 - 40.71}{-.07696} = Y_2 - 18.28$$

with numerical values. The direction cosines of this second axis differ in sign, and this trend of variation corresponds to a joint increase of one variable and decrease of others. A positive deviation of 100 units in $Y_2$ would correspond approximately to deviations of (—49, +87, —8) millimeters in carapace (length, width, height). This second principal component is thus a trend of shape variation. The direction cosines of the third principal axis also differ in sign, and the third principal component is similarly interpreted as shape variation.

The results of the analysis of size and shape variation of the present

data are summarized in Table 4. It is seen that females are much more variable than males in size and slightly more so in shape.

TABLE 4
SIZE AND SHAPE VARIATION

| | 24 Males | | | 24 Females | | |
|---|---|---|---|---|---|---|
| Principal axes | 1st (major) | 2nd (intermediate) | 3rd (minor) | 1st (major) | 2nd (intermediate) | 3rd (minor) |
| Magnitude of variance | 195.28 | 3.69 | 1.10 | 680.40 | 6.50 | 2.86 |
| % of total | 97.61 | 1.84 | 0.55 | 98.64 | 0.94 | 0.41 |
| Nature of variation | *Size* joint variation of all dimensions | *Shape* contrast of length vs. width mostly | *Shape* contrast of length vs. height mostly | *Size* joint variation of all dimensions | *Shape* contrast of length vs. width mostly | *Shape* contrast of length and width vs. height |

## SCATTER DIAGRAMS OF SIZE AND SHAPE

The principal axes of an ellipsoid are mutually perpendicular. The principal axes of equal frequency ellipsoids can therefore be used as new orthogonal coordinate axes. Heretofore the sets of measurements (length, width, height) have been interpreted as dots in the orthogonal coordinate system of variables $X = (X_1, X_2, X_3)$ or

$$X' = \begin{bmatrix} X_1 \\ X_2 \\ X_3 \end{bmatrix} \text{ in colum-vector notation.}$$

The new coordinates Y of the same dots on principal axes with direction cosines U are given by $Y' = UX'$

$$\text{or} \quad \begin{bmatrix} Y_1 \\ Y_2 \\ Y_3 \end{bmatrix} = \begin{bmatrix} U_{11} & U_{12} & U_{13} \\ U_{21} & U_{22} & U_{23} \\ U_{31} & U_{32} & U_{33} \end{bmatrix} \begin{bmatrix} X_1 \\ X_2 \\ X_3 \end{bmatrix}$$

in extended matrix notation. The operation transforming the X- into the Y-coordinates is commonly referred to as a rotation. The sets of values of the new variables have been represented in two-dimensional scatter diagrams (Figures 5 and 6). The latter constitute views of the tridimensional diagram (Figures 3 and 4) seen in the direction of principal axes.

To determine 95 per cent equal frequency ellipses of groups of dots in bivariate scatter diagrams of the elements of $Y = (Y_1, Y_2, Y_3)$, the covariance matrices of these new variables must be known. These could of course be calculated directly from the values of Y. However, it is much simpler to rotate the X-covariance matrices to the Y-axes. Thus a sample with covariance matrix W on the X-axes has covariance matrix UWU′ on the Y-axes since $Y' = UX'$. Upon rotation to the male principal axes, the male covariance matrix assumes its diagonal form, $\Lambda$, while the female covariance matrix remains non-diagonalized to the extent to which it differs from that of males. The reverse situation occurs when rotating to female principal axes.

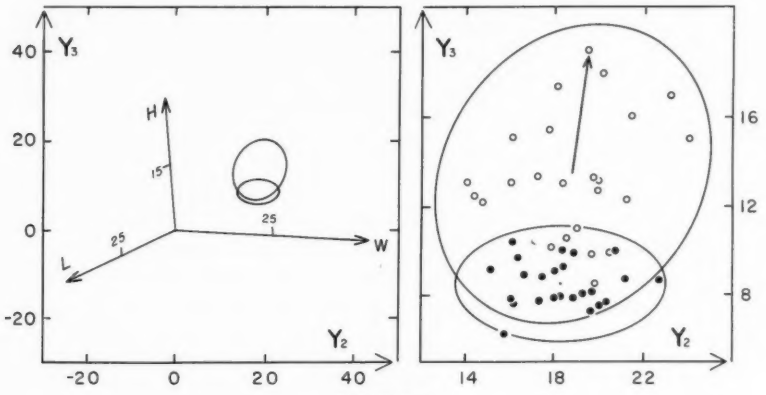


FIGURE 5
View perpendicular to the second and third principal axes of males; blown-up portion of Figure 4.

The cross sections of the male (Figure 5) and female (Figure 6) ellipsoids in the plane of $(Y_2, Y_3)$ are both flattened in the direction of height. This suggests that there is less shape variation in carapace height than in carapace length and width. This may have no biological significance, however, since the order of magnitude of carapace height is smaller than that of length and width and all dimensions are expressed in millimeters. The influence of scales of measurement on principal components is discussed by Anderson (1958: 279). Standardizing variables (Teissier, 1955: 345) is certainly often preferable when only one group of observations is considered; the standard

deviation of each variable is taken then as its unit and the covariance matrix becomes the correlation matrix. In the present case, however, this was not done as it would have led to different scales of measurement for males and females.

The cluster of female observations is clearly above that of males in Figures 5 and 6; this expresses the markedly greater carapace height of females. Although principal component analysis brings out well this difference of shape between the sexes, analyzing multivariate differences between groups of observations calls generally for discriminant functions. Discriminatory analysis leads to trends of maximum variation between groups of observations in the same way as principal components lead to trends of maximum variation within groups. The use of discriminant functions has been illustrated and discussed recently by Jolicoeur (1959). However, in the present study, the major interest lies in the nature and the magnitude of variation within local populations, and this calls for principal component rather than discriminatory analysis.

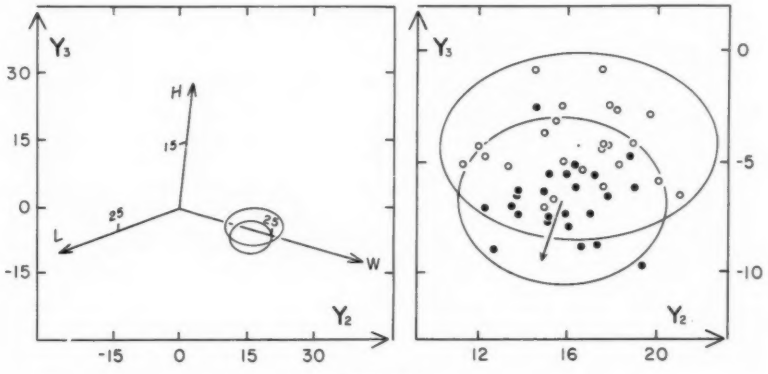


FIGURE 6
View perpendicular to the second and third principal axes of females.

## Divergence in Shape During Growth

The major axis of each sex projects merely as a point on the plane of its own second and third principal axes (Figures 5 and 6). Correspondingly, size variation is absent in the scatter diagram of each sex with respect to its second and third principal components. On the

other hand, male and female major axes are not parallel and the size variation of each sex is present with respect to the second and third principal components of the other sex. Thus large females tend to congregate upward from their mean in Figure 5 and large males downward from their mean in Figure 6.

The extent and the manner in which males and females diverge in shape as they increase in size can be judged from the projection of the major axis of each sex on the plane of the second and third axes of the other. These projections are indicated by arrows (upwards from the mean of females in Figure 5; downwards from the mean of males in Figure 6). Each arrow corresponds to a length of $\sqrt{5.99\lambda_1}$ units and its extremity can thus be visualized as the tip of an ellipsoid whose projections are the 95 per cent equal frequency ellipses represented. It is clear from this graphical analysis that there is a progressive divergence of relative carapace height between the sexes during growth. Also, carapace width appears to grow at a slightly higher rate in females.

## Conclusions

Painted Turtles exhibit considerable variation in carapace dimensions. While the general nature and the magnitude of variation can be perceived by inspection of the data, they are made much more explicit by bivariate scatter diagrams and equal frequency ellipses. When considering multiple characters, however, multivariate statistical techniques are necessary to a unified analytical approach. Applied to carapace length, width and height, principal component analysis discloses three uncorrelated and mutually perpendicular trends of variation. The first principal component corresponds to a direction of size increase and can therefore be interpreted as a growth trend. The second and third principal components correspond to disjoint variation of the various characters and are consequently interpreted as trends of shape variation. Female painted turtles vary much more than males in size and slightly more in shape. While small turtles of both sexes are of comparable size and shape, females reach a greater size and their carapace becomes relatively higher than that of males.

The divergence in shape of males and females during growth is expressed by the difference in direction between the major axes of their covariance matrices. But covariance matrices are necessarily unequal

when their principal components are not identical in direction and in length. The very fact that groups of living organisms have unequal covariance matrices can thus be of considerable biological significance since the way in which shape differences relate to size differences is of major importance in all biological disciplines directly or indirectly concerned with development and growth. Assuming covariance matrices of sets of data to be equal, as often done in theoretical statistics, would therefore frequently be unrealistic and misleading from the viewpoint of the biologist. Proper attention can be paid to growth divergences between groups of living organisms if principal component analysis is used. The latter seems the most promising method for the study of size and shape variation in complexes of biometrical characters.

The use of straight or curved lines is very common in relative growth studies and it has been customary to regard departures of data from growth curves as having little biological meaning. Such an attitude is difficult to maintain, however, when deviations of biometrical characters from growth curves are found to be correlated. The description of relative growth data in terms of several axes of variation, one of size and the others of shape, appears therefore more appropriate than their description in terms of a simple growth curve. The latter would generally constitute an over-simplification and would result in a loss of biological information.

At first, principal component analysis is most likely to be regarded by the non-mathematician as a highly arbitrary set of manipulations. Such a reaction should be readily dismissed, however, as soon as the geometrical meaning of the method is considered: principal component analysis merely leads to new angles of viewing data, angles best suited to disclose the nature and the magnitude of size and shape variation.

## Summary

The applicability of principal component analysis to size and shape variation in living organisms is illustrated by a study of male and female painted turtles. Numerical and geometrical aspects are discussed in detail and it is concluded that principal component analysis of size and shape variation would be appropriate in relative growth studies whenever multiple biometrical characters are considered.

## Acknowledgments

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# EFFECT OF CELL DENSITY ON GROWTH IN HELA CELLS[1]

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To make full use of recent advances in replicate culture techniques, it is essential that the inoculum range for optimum proliferation be known for each newly developed clone. This knowledge, along with other necessary standardizing procedures, would greatly facilitate duplication of these procedures by investigators employing cytoculture methods.

Earle et al. (1) ascertained the range of inoculum size best suited to proliferation in strain L cells (NCTC Clone #929) and Pace and Aftonomos (7) investigated the same for a clone of mouse liver cells (NCTC Clone #1469). However, no such values have been reported for the widely used human cervical carcinoma strain, HeLa (2), which has recently been cloned. The present report concerns optimum initial cell density range of this clone.

## MATERIALS AND METHODS

HeLa cells were cultured in a nutrient medium consisting of 40% human serum, 40% Earle's balanced salt solution, and 20% chick embryo extract (1:1).

Cells were cultivated in T-60 flasks as stock cultures from which they were taken for experimental purposes by careful scraping immediately following a nutrient medium change. Replicate cultures were set up in D-3.5 Carrel flasks using one ml. of medium containing the desired number of cells for each culture (7). Nuclei counts were made according to the method devised by Sanford et al. (8).

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## RESULTS

A total of 25 different tests were made with initial cell densities ranging from 2,000 to 3,500,000 cells/ml. The number of tests varied from 2 to 5 for the different cell densities and at least 50 cultures were used in each test.

The results are presented graphically in Fig. 1 which represents cell counts made every 48 hours from the beginning of the experiments. Each point is an average count for 9 cultures.

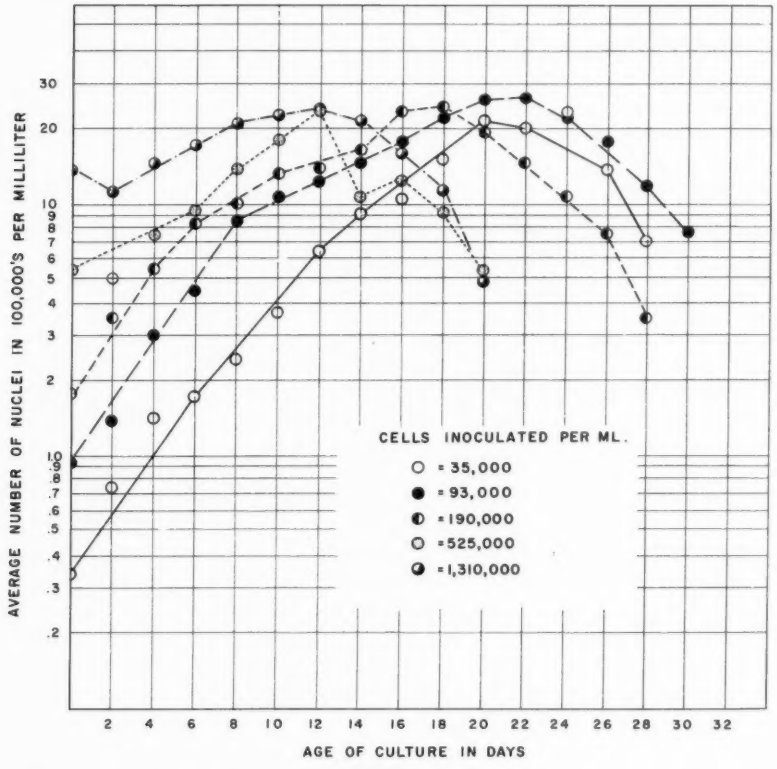


FIGURE 1

Growth of HeLa cells in cultures of different initial cell densities. Counts were made every 48 hrs. Each point on the graph is an average for 9 cultures.

Several interesting phenomena are indicated by the graph. First, it is evident that maximum cell numbers obtained are never as great as those obtained for strain L (1) or mouse liver epithelial cells (7). The liver cells sometimes reach a maximum of nearly 6 million cells/ml.; however, HeLa cells very seldom numbered more than 2.6 million cells/ml. under similar conditions.

Other results (not shown in figure) also indicate a considerable difference in the lowest initial cell density required for growth in HeLa cells, 4,000 cells/ml., as compared to 90,000/ml. for liver cells. HeLa cultures inoculated with fewer than 4,000 cells/ml. did not proliferate and those inoculated with 4,000 6,000 or 15,000 cells/ml. did not show 100% proliferation. The results obtained with these low densities are not included in the figure for this reason. However, proliferation occurred in most of these cultures and maximum numbers reached were over 2 million cells. Growth curves were not too different from those shown for cultures which had an initial cell density of 35,000 cells/ml.

Other results not included in the figure are those obtained for cultures with initial cell densities of 60,000, 150,000, 344,000, 1,075, 000 and 1,980,000 cells/ml. Growth in these cultures was almost identical to growth in those cultures represented in the figure with inocula not too greatly different from those given above. Cell increase was not evident when initial cell density was 3,000,000/ml. or greater. In fact, with this initial density the cultures gradually decreased in population from time of inoculation.

In HeLa cells, as in all other cell clones that have been investigated in this manner, the greater the initial cell density, the sooner is the maximum reached. It is also indicated that the greater the initial cell density, the greater the number of cells that die and disintegrate within the first few days.

Maximum cell number is greatest (approximately 2.5 million cells/ml.) in those cultures in which initial cell density ranges between 60,000 and 1,980,000 cells/ml. but in the latter cultures the cells soon die and disintegrate.

## Discussion

Because of the results of investigations previously made on cloned tissue cells (1, 7) as well as upon some protozoan species (3, 4, 5, 6) we are of the opinion that cells elaborate a substance which, within

limits, controls growth of the cell itself. Some of the observations noted in studying growth of cells and cell populations may be explained by assuming the existence of such a substance or substances which must be present in certain concentrations before the cells will divide and grow normally. Thus a single cell or even a few cells do not grow and flourish when placed in 1 ml of culture medium. However, in the case of HeLa cells, as few as 4,000 cells/ml. will grow and produce a population of 2 million or more cells. However, this maximum is not reached until 32 days following inoculation.

Present evidence makes it difficult to draw conclusions as to the difference in required initial cell density in HeLa cells as compared to that of strain L and liver cells. In both these latter strains, proliferation failed if cultures were started with fewer than 90,000 cells/ml. Perhaps the difference lies in the type of epithelium represented by the HeLa cells. Future investigations may clarify this question.

## Summary

1. A study of optimum initial cell density for strain HeLa has been made.

2. Cultures with initial cell densities of less than 4,000 cells/ml. do not survive. Indications are that cells will proliferate in cultures with inocula of 4,000 to 15,000 cells/ml. but growth under these conditions is uncertain.

3. Initial cell densities of 35,000 to 120,000/per ml. do not reach their maximum cell numbers for approximately 3 weeks. Those cultures inoculated with 150,000 to 1,180,000 cells/ml. reach maximum numbers within 12 to 18 days.

4. Under the conditions of these experiments, it is indicated that between 2,500,000 and 2,800,000 cells/ml. represent maximum cell population for the HeLa strain.

5. When cells attain maximum numbers within a culture, there is a rapid decrease in number—the greater the density the greater and more rapid this decrease.

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*Growth*, 1960, **24**, 361-383.

# HYPOXIA AND MALDEVELOPMENT OF EARLY ALAR-PLATE TISSUE IN MID- AND FORE-BRAIN REGIONS OF THE CHICK EMBRYO*

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(Received August 31, 1960.)

## INTRODUCTION

### 1. *Orientation*

The observations described in this presentation are a small part of a comprehensive study of cell- and tissue-behavior during morphogenesis of the head (cephalogenesis) in the chick embryo. In the particular study described here hypoxia and a mechanical separation of tissue were employed as analytical agents in determination of developmental behavior. Exposing embryos to these privations aroused, in many instances, the formation of a brain-head anomaly known as pseudoencephaly. An increased incidence of lethality was a common result of exposure to low partial pressures of $0_2$.

A special feature of early cephalogenesis is a pronounced growth and extension upward and forward from more posterior tissues on the part of the individuating brain and other components of the developing head. As this cephalogenic activity progresses the future brain tissue is folded into a primitive tube. The final step in the tubulation of neural tissue involves a fusion of the lateral edges of the folding material as these edges meet in the mid-dorsal line. This area of fusion of cellular layers forms the rudiment of the roof-plate or dorsal wall of the brain and spinal cord. The condition of the neural tube which results from failure of the lateral margins of the developing tube to meet and fuse dorsally is called asyntaxia dorsalis. The latter term is general and applies to any region of the central nervous system which fails to close dorsally, but the word acleiencephaly (Ancel', 55) applies to failure of the brain region to complete the tubulation process. In this paper the word pseudoencephaly designates

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a brain-head condition which arises either from the acleiencephalic state or from weak fusion of the roof-plate area of the developing brain. As pseudoencephaly develops, the lateral walls of the affected area of the brain undergo movements of eversion to form a bilateral, monstrous affair which opens to the exterior as shown in figs, 1 & 2. In most cases dorsal craniogenesis fails, and the internal aspect of the brain in the affected area becomes a part of the head's exterior. The degree of eversion of the brain walls which occurs during the development of this catastrophic condition varies according to the degree of openness of the developing brain. If acleiencephaly is slight, or if a weak area of the roof-plate exists, the eversion movements will be extensive; if acleiencephaly is marked, the movements of eversion will be less.

2. *Purpose of investigation*

In the course of previous work with low partial pressures of $O_2$ in relation to chick development occasional acleiencephalic and pseudoencephalic embryos were observed. These conditions occur in the chick in rare instances under normal air conditions. Also, pseudoencephaly is one of the devastating, teratoid conditions which arises on rare occasions in human development. (See Ingraham, '44). Studies related to activities responsible for this anomalous condition therefore are of interest.

The main purpose of this investigation, however, was not to elicit abnormality. Rather, its aim was to observe morphogenetic behavior in the chick embryo during early head organization in the same minimal $O_2$ mixture which appears adequate for movement of head-organizer material forward during the cephalic-projection stage of gastrulation. This mixture has been shown to be approximately 6% $O_2$, 0.5 $CO_2$, remainder $N_2$. (Nelson '58[a]). The reason for this comparison, and also for the use of 8%-$O_2$ and 10%-$O_2$ mixtures, was to determine, under embryo-egg relations, the increase in $O_2$ need which head-organizer material arouses in the overlying ectoderm (see p. 380) as it initiates normal head development and organization. A further objective was to compare the demand for $O_2$ in the brain area with that of the developing neural tube situated more posteriorly. As acleiencephaly and pseudoencephaly are easily produced under certain partial pressures of $O_2$, effort was made to determine factors concerned with their development to gain insight into tissue behavior and $O_2$ requirements during normal brain formation.

3. *Other observations of induced acleiencephaly and pseudoencephaly*

Pseudoencephaly in the mouse embryo was studied carefully by Bonnevie, '36. As a result of this study, Bonnevie attributed this condition, together with a considerable number of embryonic deaths in the particular strain of mice under observation, to a recessive (?) gene. Consequently, she regarded this genic condition as a probable part of the hereditary system in this strain. Furthermore, Snell, Bodemann, and Hollander, '34, experimentally obtained pseudoencephalic offspring from X-radiated male mice mated with normal, non-radiated females. Some of these pseudoencephalic embryos reached full term, but were stillborn. A number of embryos which experienced early death were obtained by these investigators along with the pseudoencephalics. On the other hand, Snell and Picken, '35 using a similar technique, obtained another stock of mice whose offspring developed pseudoencephaly with a smaller number of inviable litter mates. Snell and his co-workers suggested that the pseudoencephaly in their experimental stocks was the result of translocations between segments of chromosomes.

The foregoing examples of pseudoencephaly which appear to result from disturbed chromosomal states are prezygotically determined. That is, they are present in the germplasm previous to fertilization. It is possible that the same basic embryological mechanism is disturbed by the recessive (?) gene and by the chromosomal translocations induced by X-radiation of the male germ cells.

In addition to these examples which suggest that chromosomal aberrations of sorts may be responsible for embryological conditions which result in pseudoencephaly, there are experimental studies which indicate that environmental agents applied to the embryo during a specific, crucial period of its development can induce developmental disharmony which leads to this condition. For example, Kaven, '38, Russell, '50, Wilson, Jordan and Brent '53, X-rayed pregnant mice during the 7-8 day period of gestation and found that the 8th day of development was a sensitive period favorable for the inducement of pseudoencephaly and other brain abnormalities. Hamburgh, '54, induced acleiencephaly with subsequent pseudoencephaly in mouse embryos by injecting the mother with a Trypan Blue solution on the 8th day of pregnancy, and Ingalls, et al, '50, '52, stimulated brain anomalies in the mouse by subjecting the mother to low $O_2$ pressures

on the 8th day of pregnancy. In the rat embryo, Wilson & Karr, '51, and Hicks, '53, elicited brain anomalies by X-radiation of the mother during the 9-10 day gestation period. Davis, '44, irradiated chick embryos from the primitive streak to 8 somite stages with monochromatic ultraviolet light effecting a marked inhibition of tube formation. Lutz and Lutz-Osterlag, '58, produced similar results in the brain area with ultrasonic treatment of chick blastoderms during the late primitive-streak and head-processes stages cultured in a special saline-nutrient medium. Ancel, '55, produced acleiencephaly in the chick embryo in various areas of the brain by injecting small amounts of ricine, or larger quantities of trypaflavine, onto the cephalic end of the embryo during the early part of the second incubation day. Buchner, Rubsaamen, and Rothwill, '51; Mushett, '53; Naujoks, '53; and Buchner, '55, induced brain deformities among many other teratoid conditions by subjecting chick embryos to low $O_2$ pressures for varying periods during the first and second incubation days.

## Material and Methods

Eggs from the White Leghorn variety were used. They were kept at room temperature for 24 hrs. before incubation. The incubating temperature was 37.5°—38°C. The eggs used in these experiments were laid during the period from November to June in the years 1958-1960.

As mentioned previously, two different procedures were utilized in this study.

*Procedure 1.* During the course of the study reported previously (Nelsen, '58[a]) it was observed that eggs exposed sufficiently during their second-day of incubation to 6%-$O_2$ and 8%-$O_2$ mixtures developed anomalous conditions in the brain area. This work has been repeated more extensively using 6%, 8%, and 10% $O_2$ together with a longer post-exposure period of incubation in a forced-draft incubator.

The manner of exposure was the same as previously described, (Nelsen, '58[a]), with the exception that a two hour warm-up period in a forced-draft incubator was inserted into the procedure for the experimental eggs before they were placed in the exposure chamber. This warm-up period removed the probability that the experimental eggs produced a cooler temperature within the exposure chamber during the earlier portion of the exposure period, and hence, would

not be incubated at the 37.5°—38°C as long as the control eggs. When the eggs were removed from the chamber, they were placed at a distance of 18 inches directly below the fan in the forced-draft incubator. The latter procedure permitted the recently-exposed eggs to be washed with rapidly-flowing, warm air to establish normal-air conditions within the egg's substance as rapidly as possible. Before connecting the chamber to the latex tubing conveying the gas from the repository cylinder, the gas was permitted to flow through the accessory apparatus for 30 minutes to wash out other gaseous mixtures.

An equal number of control eggs were run with the experimental eggs. The control eggs were kept in the forced-draft incubator, the same incubator which housed the chamber. Six experimental and six control eggs were run in each experiment. Following the period of exposure experimental and control eggs were candled daily to note survival.

*Procedure 2.* The purpose of the observations depending upon this procedure was to observe the morphogenetic movement of the walls of mechanically reopened brains and to compare these results with those obtained with hypoxia. In these experiments the closed encephalon was reopened dorsally at approximately stages 11 and 12 of Hamburger and Hamilton, '51. Two methods of handling the eggs during the operational procedure were used.

a. *Egg-shell method.* Eggs incubated for the general period of time necessary to produce the required embryonic stages where opened carefully under sterile conditions. In doing so, an area of the shell about 2.5 cm. square was removed by means of a fine saw and scissors. The blastoderm was lightly stained with a thin strip of gelatin impregnated with neutral red. If the embryo was in the correct developmental stage, a particular segment (or segments) of the brain was opened in the mid-dorsal line with a sterile, fine needle made from a small glass rod or from thin tungsten wire. After the operation the egg was closed by fastening a disc of sterile egg shell over the opening by means of melted paraffin. Incubation was continued for varying periods up to the eighth day in a forced-draft incubator.

b. *Sterile beaker method.* Following the requisite incubation period, the yolk, and its associated albumen, was gently rolled out into a sterile 30 cc. beaker. By means of sterile instruments the yolk was rotated to permit the blastoderm to appear uppermost if it did not

rotate by itself, and the embryo was stained as above. After completion of the operation the beaker was placed in a sterile, humidified glass chamber. The latter was inserted within a still-air incubator for varying periods up to the eighth day.

Four areas of the brain were opened in this study, namely, a) the entire brain from the anterior end of the rhombencephalon forward; b) mesencephalon and prosencephalon; c) mesencephalon; and d) prosencephalon.

## Results

1. *Exposures to 6% $O_2$, 0.5% $CO_2$, remainder $N_2$.*

   a. *Exposure: 22 hrs. following 2 hr. warm-up period.*

      Results: *Experimental. Thirty-six eggs.*

      Three infertile; 2 died between exposure and 5th day; 1 died 7th day; 1 died 8th day; 29 living and normal in appearance on day 10. Size of embryos comparable to controls.

Note: A test run to determine degree of development following exposure for 22 hrs. to this gaseous mixture was made. This run consisted of 12 eggs divided into two exposures of 6 eggs each. The following stages were found to be present at the end of the 22-hr. exposure: 1 infertile; 1 early-streak; 3 broad-streak; 1 approaching definitive-streak; 5 definitive-streak; 1 early head-process. The results obtained in this test run indicate that the embryos after 22 hrs. of exposure were not subjected to hypoxia after the appearance of head- and neural-fold stages.

*Control. Thirty-six eggs.*

Two infertile; 1 died 5th day; 1 died 8th day; 32 living on day 10.

   b. *Exposure: 26 hrs. following 2 hr. warm-up period.*

      *Results: Experimental. Thirty six eggs.*

      Two infertile; 6 died between exposure and 9th day; 28 living on 10th day. Slight retardation of development evident in 9 embryos; 1 embryo anophthalmic; 2 embryos with one eye reduced in size.

Note: A test run of 24 eggs to determine degree of development after 26 hrs. of exposure to this level of $O_2$ presented the following

stages: 2 infertile; 7 definitive streak; 3 eary head-process; 10 head-process; 1 head-fold with first pair of somites forming; 1 early head-rudiment stage with first and second pairs of somites formed.

*Control. Thirty-six eggs.*

Four infertile; 2 died before 3rd day; 1 died 6th day; 29 living and normal in appearance on 10th day.

Is is evident from experiments 1,a and 1,b that the morphogenetic movement of cells during the first phase of gastrulation, in which cell groups are projected forward to establish the basic cellular pattern necessary for organizing the future head region, are not disturbed too much by this degree of hypoxia. That the level of $O_2$ in 1,b is not optimum is demonstrated by a slight lag in the test-run eggs of 1,b. That is, a number of the head-process embryos in this experiment normally should have achieved head-fold and early somite stages, and most of the definitive streak embryos should have been in the head-process stage.

c. *Exposure: 32 hrs. following 2 hr. warm-up period.*

*Results: Experimental. Thirty-six eggs.*

Four infertile; 23 embryos died between exposure and 6th day; 3 died 7th day; 2 died 9th day; 4 were living when eggs were opened on the 10th day. Of these four embryos 1 was acephalic, neck and part of hind-brain present; 1 was pseudoencephalic involving diencephalic and telencephalic areas; 2 were normal. Among the 27 embryos dying from 2nd to 9th days, 11 were much retarded and apparently normal with the exception of 3 embryos whose heads were badly mangled and non-descript. The remaining 16 of the 27 exhibited various stages of pseudoencephaly, including 9 embryos whose entire brain was involved from the anterior myelencephalon forward, 4 embryos with the diencephalon and telencephalon involved, and the remaining 3 embryos with the mesencephalic area pseudoencephalic. Hemorrhaging from surface of the pseudoencephalic areas was observed in three of the older embryos. Retarded development conspicuous with the exception of one of the two embryos living and normal in appearance on the 10th day.

*Control. Thirty-six eggs.*

Three infertile; 3 died before 6th day; 30 living and normal on the 10th day.

Note: Three test runs of six eggs each exposed to the 6%-$O_2$ mixture demonstrated that the experimental embryos lagged behind the control embryos. Test runs of the controls for the first 32 hrs. of incubation presented embryos from the early somite stages to ten somites, while the experimental test runs produced a few embryos in definitive streak and head-process stages but most of these embryos were in the somite stages up to the fifth-somite stage. The head regions of the latter embryos manifested irregularities (see description below) but the truncal regions in all appeared normal.

Embryos exposed to the 6%-$O_2$ mixture for the first 32 hrs. of incubation presented the following conditions of development: the early somite embryos apparently were normal, but most of the embryos in the four and five somite stages possessed neural folds which twisted laterally. Some of these embryos had stunted head outgrowths, but in others the outgrowth appeared normal in size. In one of the older embryos the neural tube of the brain area was abortive although the forming tube in the somitic region assumed a normal appearance. A stunted head outgrowth in some of the embryos, together with an abnormal form of the neural folds, suggests that both neural and epidermal ectoderm were suppressed by this low partial pressure of $O_2$. The ventral aspect of three embryos possessed abortive stages of the fore-gut rudiment indicating an adverse effect upon the developing gut.

2. *Exposures to 8% $O_2$, 0.5% $CO_2$, remainder $N_2$.*

   a. *Exposure: 24 hrs. following 2 hr. warm-up period.*

   *Results: Experimental. Twenty-four eggs.*

   Three infertile; 4 died between end of exposure and 5th day; remainder living and normal on 10th day.

   *Control. Twenty-four eggs.*

   Two infertile; 1 blastoderm degenerated early; 2 died between 3rd and 5th days; 1 died 8th day; remainder normal on 10th day.

b. *Exposure: 32 hrs. following 2 hr. warm-up period.*

*Results: Experimental. Thirty-six eggs.*

One infertile; 1 early degenerate; 5 died before the 5th day; 3 died between exposure and day 10; 26 survived to day 10. Of the latter group, two were rumpless but otherwise normal, 7 had reduced ophthalmic structures and three of these were also retarded in development, 8 were normal in appearance and size, and the remaining 9 were retarded but apparently normal.

*Control. Thirty-six eggs.*

One infertile; 1 died 2nd day; 3 more dead 5th day; 31 living day 10, all normal in size and appearance.

c. *Exposure: 40 hours following 2 hr. warm-up period.*

*Results: Experimental. Forty-two eggs.*

Three infertile; 24 died 2nd to 5th days; 4 pseudoencephalics described below survived beyond the 5th day; 11 other embryos survived to day 10. The latter 11 embryos, although somewhat retarded, were normal in body appearance with the exception of ophthalmic deficiencies in some.

*Control. Forty-two eggs.*

Two infertile; 3 died during first three incubation days; 1 died 6th day; 36 normal on day 10.

Of the four pseudoencephalic embryos which survived beyond the 5th day of incubation, one died on 6th day, and one egg candled on the 7th incubation day indicated vascular changes in the blastoderm and therefore the egg was opened. The embryo was found to be hemorrhaging from papilla-like elevations on the surface of the open-brain area. A third embryo was dead when the egg was candled on the 8th incubation day. This embryo had hemorrhaged badly and the amnion was filled with bloody fluid. One pseudoencephalic embryo was obtained from eggs opened on the 10th day of incubation and showed, in the fresh condition, small, irregular, papilla-like red areas on the everted brain surface although actual hemorrhage was absent. Acleiencephaly and the resulting pseudoencephaly involves much of the brain in embryos exposed to the 6%-$O_2$ mixture, but in embryos

exposed to the 8%-$O_2$ mixture the mesencephalon principally is involved. See figs. 1 & 2. Note: Test runs of eggs exposed to the 8%-$O_2$ mixture for 40 hrs. demonstrated that the contained embryos were in a retarded state of development. These experimental embryos were in the developmental range of 7-12 somites whereas embryos in control eggs were in the 15-22 somite stages.

3. *Exposures to 10%-$O_2$ mixture.*

Exposures to 10% $O_2$, 0.5 $CO_2$, and remainder $N_2$ beginning at end of 2 hr. warm-up period and continuing to early part of 3rd day failed to disturb tubulation of the central nervous system. However, two microophthalmic and one unilateral anophthalmic embryos appeared in 36 experimental eggs. All embryos were normal in size on 10th day of incubation. Six embryos died between the end of the exposure and the 10th day.

4. *Histogenic features of the acleiencephalic regions of the chick's brain induced by hypoxia.*

The development of neural tissue including ependymal zone mitoses, cellular elongation, and general increase in thickness of the tubular wall proceeds normally in the open-brain area up to about the beginning of the 6th day of incubation. During the 6th day of incubation changes in histogenic behavior begin to occur, and from this time on the normal pattern of differentiation is disturbed including vasculogenesis. In the condition shown in figs. *1, 2* & *3* the pattern of neurogenesis is completely distorted, and an irregular tumor-like growth of tissue is present. Some of the blood vessels develop sinus-like structures which contact the surface where they ultimately rupture. See fig. *4*.

5. *Acleiencephalic conditions produced operatively.*

In the majority of instances when the mesencephalon or the prosencephalon were opened reclosure occurred and the brain region of the embryo appeared normal later. On the other hand, when the brain from the anterior rhombencephalon forward was opened the acleiencephalic condition persisted in approximately 30% of the embryos. As this phase of the study is still in progress in relation to other work dealing with the influence of the open neurocoel and disturbed neurocoelic conditions on neurogenesis in the brain region,

further details will not be mentioned here. It is sufficient to say for present purposes that general morphological and histological results of the pseudoencephalic brains in embryos produced by the operative method who lived beyond the 6th incubation day were similar to be pseudoencephalics developed as a result of exposure to 6%-and 8%-$O_2$ mixtures. Both of these methods elicited brains with the inner, ependymal surface of the neural tissue thrown into irregular folds with distortion of the histological pattern of development. The folding and distortion of the inner surface of the brain, now exposed to the exterior, becomes more pronounced as development progresses. It is evident, therefore, that acleiencephaly achieved either by mechanically reopening the mid-dorsal area of the recently closed brain or by subjecting the embryo to hypoxia produces a similar pseudoencephalic end.

One may parenthesize here that the above experiment on reopening of the primitive brain demonstrates that destruction of simple, anatomic relations may disturb forces within a developing structure to the extent of destroying its individuation and the individual. It also emphasizes that biophysical and mechanical factors external to, and apart from, a cellular area, may be of utmost importance to the proper differentiation of the cells within the area. (See also Fowler, '53, and Coulombre and Coulombre, '58).

## Discussion and Summary

1. *Pseudoencephaly—an anomaly associated with retarded development.*

In the mouse embryo the anterior end of the notochord becomes firmly attached to the under side of the presumptive brain tissue during gastrulation. The anterior end of the notochord normally separates from brain tissue before the 7- to 8-day period of gestation. In the study made by Bonnevie, '36, she observed that the anterior chorda material in two embryos was still attached to the overlying brain tissue as late as the 8th day of gestation. This slowness or retardation of development revealed by the late separation of the notochord from the brain tissue was the first indication of abnormality that Bonnevie was able to detect in the pseudoencephalic stock which she studied. The importance of the slow separation of the chorda

## PLATE I

Photographs of pseudoencephalic embryo obtained from egg after exposure to 8% $O_2$ for the first 40 hrs. of incubation following 2 hr. warm-up period. Incubation continued in forced-draft incubation to the 10th day.

Figure 1—Right view of embryo. Observe microopthalmic condition of eye. Tissue of brain exposed is lobulated and similar bilaterally.

Figure 2—Left view of same embryo shown in fig. 1. Eye normal in size.

1

2

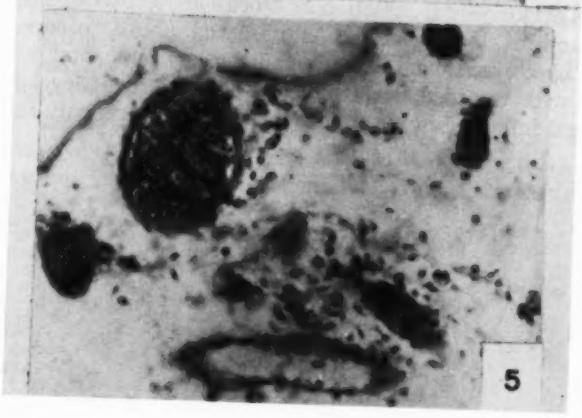
## PLATE II

Figure 3—Dorsal-view photograph of pseudoencephalic brain in embryo exposed to 6% $O_2$ for first 32 hrs. after 2 hr. warm-up period. Incubation continued in forced-drafts incubator until the 8th day. The entire brain is involved from the anterior rhombencephalon through the telencephalic region.

Figures 4 & 5—Photomicrographs of sections of pseudoencephalic brain showing large blood vessels near surface of the folding tissue shown in Figs. 1-3. These blood vessels eventually rupture to the surface and the embryo hemorrhages to death.

3

4



5

material which Bonnevie observed is that it indicates a general retardation of the normal developmental sequence. In addition, in later embryos Bonnevie observed irregular curvatures or kinking of the posterior brain region and a weakened closure of the roof-plate in the caudal mesencephalon. It is evident that there is a general similarity between the precursive conditions to pseudoencephaly observed by Bonnevie in the mouse embryo and those found in the chick embryo by the author. However, in the actual formation of the pseudoencephalic condition Bonnevie emphasized increased tension by the Trigeminal Nerves and the bending movements associated with the development of the Cephalic Flexure, whereas the author contends that in the chick embryo eversion of the brain wall results from growth and differentiation in the alar-plate areas of the mid- and fore-brain segments of the developing brain.

The pseudoencephalic distortion described by Bonnevie and the similar malformation associated with retarded development induced by X-radiation of the male germ cells as reported by Snell, Bodemann and Hollander, '34, are examples of hereditary maldevelopment. The condition of rumplessness in the fowl, (see p. 379) in which Landauer and Bliss, '46, were able to select high and low incidence groups whose eggs when injected with insulin produced embryos with this hapless condition, may be classified as teratosis dependent upon genic and environmental factors. On the other hand, hypoxia-induced pseudoencephaly in the chick embryo, or brain terata aroused in the mouse embryo by injecting the mother on the 8th day of pregnancy with Trypan Blue are examples of disharmonies excited by environmental agents. (See Hamburg, '52 and Waddington and Carter, '53). Whether induced by hereditary influences or by environmental conditions, anomalisms often are associated with retarded development in various degrees. Stockard, '21, and Wolff, '48, consider depressed metabolic mechanisms which result in retarded development—and in extreme cases in developmental cessation or block—to be part of the causative background of teratogenesis. As observed above, pseudoencephaly fostered by chromosomal deviations or by environmental means is associated with retarded development. Does retarded development act as a causal influence in hypoxia-induced pseudoencephaly in the chick embryo?

2. *Developmental retardation as a factor responsible for pseudoencephaly.*

The position that developmental retardation may play an important role in the development of pseudoencephaly may be challenged by some students of teratology. (See Zwilling, '55, p. 706). The present study, however, suggests that depressive effects exerted by hypoxia upon metabolic mechanisms differ in respect to 1) the basic tissues, and 2) the particular morphogenetic process involved. To approach these matters more specifically, let us correlate morphogenetic movement with $O_2$ requirement during the earlier aspects of gastrulation and cephalic emergence.

As gastrulation gets underway in the chick embryo there is a cephalic projection, below the overlying ectoderm and along the median plane, of entoderm, prechordal plate mesoderm and notochordal cells. This forward progression of cellular materials along the median zone is associated with a more lateral movement of other mesodermal cells. (See Nelsen, '58[b] for discussion and references). These movements, in the form of flattened cell-masses, establish the head-process stage of gastrulation and arrange cellular areas requisite for the basic needs of cephalogenesis. This gastrulative behavior and placement of cells proceeds well in an air mixture containing 6% $O_2$ and 0.5% $CO_2$. However, as head realization begins new morphogenetic events arise which entail the elevation and folding of cellular layers associated with extension forward. Activities of this nature particularly are pronounced in the entoderm of the future foregut and in the epidermal and neural-plate ectoderm. The earlier stages of activity involving these new movements do not appear to be affected by the 6% $O_2$ level, but if cephalogenesis is allowed to continue in this percentage of $O_2$ embryogenesis becomes depressed and ultimately is blocked. It also appears that the early fore-gut rudiment in the forming head is hampered less than are the foldings of the neural and epidermal ectoderm, while the mesoderm, including the developing heart which is closely associated with early head formation, is disturbed least. If the metabolic block is not permitted to continue too long, the embryo may be resuscitated by exposure to normal-air conditions. Development then proceeds in most cases but in a retarded manner. In some of these redeemed embryos the head region appears normal, but in many there are anomalous conditions particularly of

the anterior part of the brain including the eyes. Acleiencephalic and pseudoencephlic deformaties are prevalent.

If the above pseudoencephalic brains are compared with those which were formed in the experiments in which the brain was reopened by longitudinal severance of the roof-plate zone it becomes evident that the same pseudoencephalic ends were obtained in the two sets of experiments. (see p. 370, 5) The close similarity between these two series of results suggests that the real factor which disturbed later brain development in both groups was a weak or non-fused roof-plate zone. This conclusion leads to a consideration of the possible influence of retarded development upon roof-plate formation.

Of prime importance is the real possibility that retardation which effects a slowing of the tubulation process may foster biochemical changes in the neural fold tissue, including the dorsal edges of the folds—changes which are foreign to the conditions present when tubulation proceeds at its normal rate. This altered state in the dorsal edges of the neural folds could lead to weak or non-fusion of the roof-plate material when development proceeds again under favorable conditions. A second aspect of the problem is the kinking or twisting of the neural folds referred to on page 368. Retarded development, for example, could hinder the normal extension of the notochord or of the expansion of the epidermal ectoderm and hence arouse forces which would hinder the forward extension of the neural tube with subsequent enforcement of a twisting effect upon the neural folds. The twisted neural folds could then act as barriers to firm fusion of roof-plate cells. Because of these possibilities, there is good reason for the view that retardation may be an influential factor in abnormal roof-plate formation and the subsequent development of pseudoencephaly.

Studies concerned with suppressed metabolic mechanisms and subsequent retarded development is one of the more important facets of embryological research. The precise factors involved in the origin of these depressed mechanisms are largely unknown. As pointed out by Brachet, '60, p. 225, the possibilities in this area "are obviously tremendous."

3. *Regional sensitivity of the neural tube to $O_2$ lack.*

The developing brain is influenced by a lowering of $O_2$ pressure particularly in those areas which are destined to be almost completely

or to a considerable extent composed of alar-plate material. That is, the developing fore- and mid-brain areas. On the other hand, the hind-brain and neural tube of the trunk region are developmentally less responsive to hypoxia. In the developing caudal or tail area the appearance of rumplessness suggests that metabolic troubles again appear in the presence of lowered $O_2$ pressure.

4. *Hypoxia and the emerging dorsal axial system.*

It is apparent that the nodal area of the primitive streak is able to function in a partial pressure of $O_2$ too low to sustain normal development in the head region. Some of the node's ability to function in $O_2$ pressures lower than the $O_2$ requirements necessary for cephalogenesis may be due to the character of the tissue involved in the node's activities, and also, to the type of cell movement in and from the nodal area. A more probable factor, however, is that the complex of enzyme systems operative in the nodal area can function under anaerobic conditions and also through direct use of $O_2$ in acquiring energy to support activity. Evidence for this behavior is presented by the work of Spratt, '58. In this study, the node appears more able to reduce tetrazole under anaerobic than aerobic conditions in the presence of a variety of substrate substances. It is evident also from the data obtained by Spratt that the brain and spinal cord, on the other hand, rely more upon aerobic than anaerobic activities. The results obtained in the present study suggest that the differentiating alar-plate tissue of fore- and mid-brain regions is more susceptible to the malevolent effects of low partial pressures of $O_2$ than are other areas of the forming central nervous system, with the possible exception of that in the tail-bud rudiment with its associated tissues. Suppression of tail-bud development may occur producing a rumpless condition when lowered partial pressures of $O_2$ are present in the environment. (Consult discussion 3 and Experiment 2,b). In this connection, Landauer and Bliss, '46, and Landauer, '47, found that insulin injected into the egg during the first 60 hrs. of incubation effects a high incidence of rumplessness, the highest incidence appearing when the injections were made at the beginning of the second day. Furthermore, Landauer and Rhoades, '52, offered evidence that insulin probably interferes with anaerobic degradation of carbohydrate material, and Spratt, '58, reported marked ability of early tail-bud cells to reduce the indicators used. It thus becomes apparent that the developing tail-bud region

possesses exceptionally active metabolic mechanisms, and that it uses aerobic and anaerobic avenues in achieving needed energy.

In summary, it may be stated that the early development of the chick embryo presents three phases of morphogenetic activity relative to $O_2$ need. As described previously, Nelsen '58[a] and '58[b], early pregastrular development can proceed in the presence of low pressures of $O_2$. The need for $O_2$ increases as the streak is formed, and when the definitive-streak state is reached and gastrulation begins, the needed $O_2$ level rises to approximately 6% $O_2$ together with 0.5% $CO_2$ in the mixture. As the head begins to evolve, dependence upon $O_2$ again is increased and a mixture containing $O_2$ somewhat in excess of 10% with 0.5% $CO_2$ is necessary for adequate development of the anterior-brain areas through stages 11 and 12 of Hamburger and Hamilton, '51. As indicated in the work of Barth and Sze, '51, the presence of organizing tissue in intimate contact with the ectoderm undergoing neuralization is an incitant which increases $O_2$ usage by the ectoderm as it becomes neuralized. If the assumption is made that a stimulating action for $O_2$ use is more intense in the anterior region of the developing neural plate (i.e., the future fore- and mid-brain areas) of the chick embryo than it is in the future hind-brain region an initial difference in the metabolism and differentiation of the two zones would, in this manner, be established.

## Conclusions

1. Eggs incubated in an air mixture of 6% $O_2$, 0.5% $CO_2$, the remainder $N_2$ for the first 22 hrs. of incubation at a temperature of 37.5°C-38°C experience normal development to day 10 of incubation.

2. The same mixture for the first 26 hrs. of incubation has a slight inhibiting effect on some embryos. Eye defects appear in a few 10-day embryos.

3. The same mixture during the first 32 hrs. of incubation proves harmful. Following exposure, lethality is high with acleiencephaly and pseudoencephaly appearing in many embryos. A small number apparently escape the depressing influence and appear normal on day 10.

4. In this mixture the behavior of the nodal area and the early development of the truncal region are superficially normal to the 32 hr. limit of incubation as exemplified by opening eggs immediately following the exposure.

5. Eggs incubated in an air mixture composed of 8% $O_2$, 0.5% $CO_2$, the remainder $N_2$ for the first 24 hrs. are not affected noticeably at day 10 of incubation. Those so exposed for the first 32 hrs. reveal subsequent adverse effects. Lethality is increased and embryos surviving to day 10 exhibit retarded development in many; eye defects in a few. Small incidence of rumplessness may occur.

6. Embryos in eggs incubated in the above mixture for the first 40 hrs. are injured. Lethality high before day 10, and retarded development present in most day-10 survivors. Incidence of pseudoencephaly much reduced, and it involves principally the mesencephalon.

7. Pseudoencephaly induced by hypoxia probably is due to depression of the metabolic rate which affects closing of the roof-plate area of the brain. Influence upon roof-plate closure may be mediated through retarded development and/or by direct affect.

8. Failure of adequate extension forward by the head rudiment, possibly due to inadequate development of the epidermal ectoderm, may be a factor in the formation of brain irregularities with subsequent head distortion and pseudoencephaly.

9. A mechanical reopening of the roof-plate zone of the primitive brain shortly after dorsal-wall formation mimics the results leading to pseudoencephaly after exposures to 6%- and 8%-$O_2$ mixtures.

10. Low partial pressures of $O_2$ affect the developing alar-plate tissues of the mid- and fore-brain regions probably because these tissues depend largely upon aerobic and less upon anaerobic activities.

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# THE GROWTH-REPRODUCTION CYCLE CLOSING PHASE

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The growth-reproduction cycle has developed by stages. Three earlier papers brought the female and male paths to points 7 and 9 (Fig. 1) respectively. The first (1) introduced minor growth (0-1), major growth (1-3-4 for female and 1-3-9 for male) and the reproduction loop (2-3-4-5-6). The second paper (2) added the path of capacity recovery (6-7), brought out the significance of path junctures as points of biological change and computed their time positions. In the third paper (3), the first and second were brought together for needed simplification of development.

A method of describing a particular female cycle pattern—using figures representing age at point 1 and years between points 1 and 4—was devised and a pattern—6.75-17.9—was selected for computation purposes (3, p. 257). Evidence in support of the cycle was noted (3, p. 258).

It now becomes desirable to divide the cycle into three phases. That part comprising minor growth will be called the minor phase. That part comprising major growth and associated paths up to points 7 and 9 will be called the major phase. Point 1, joining these two phases becomes the first point of biological change.

It was previously pointed out (3, p. 261) that point 1 is a point of stress—an indication of biological change. Corroborating evidence of this is now found in a report made on the study of 160 children by the Child Research Council of Denver (4, p. 74).

The report states: "Studies conducted by Arnold Hilden indicate that a majority of the children are unable to function at their best in intelligence tests in the period from age 5 to age 8."

The mean of "age 5 to age 8" is age 6.5. This compares with age 6.75 of the selected cycle pattern. Thus is indicated as was indicated previously in connection with the work of Dahlberg (3, p. 258) (a) a biological change as brought out by the cycle (but previously

unrecognized) in a position (b) substantially the same as that indicated by the cycle.

The third phase, that lying beyond points 7 and 9 will be called the closing phase. It is this phase—especially in its application to the human being—which we now wish to consider.

Food capacity at any point of the cycle is the amount (mass) of food which can be efficiently used in a unit of time by the organism at that point. The unit of measure of food capacity, f, has, for convenience, been given a value of one-fourth of the food capacity at point 4.

Point 0 is a mathematical representation of the beginning of life. It is a point of zero mass and zero food capacity. Mathematically, the cycle will close at point 0—again at zero mass and zero food capacity. (In the case of the human being, such a reduction to zero during life is obviously impossible and this fact precludes the possibility of life continuing all the way to point 0).

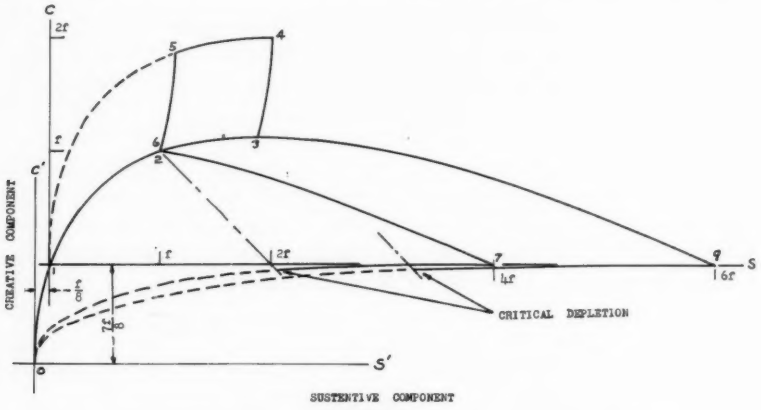


FIGURE 1
C-S Diagram Showing Closed Cycle and Paths to Points of Critical Depletion.

As shown in Fig. 1, the closing paths would be tangent to the sustentive axis S at point 7 or at point 9, as the case may be, and tangent to the vertical axis C′ at point 0. This condition of tangency places the closing paths in the same class as path 1-4 and projecting 1-4 into the closing phase results in the paths 7-0 and 9-0. (Such a

part played by path 1-4 is not surprising since, in the case of the simplest cycle (3, p. 248), path 1-4 serves as both the reproduction path and the closing path).

The area between the male growth path 1-3-9 and the S-axis, being the product of creative component and a function of time, is a measure of the mass produced during male major growth. Similarly, the area between the minor growth path and the axis S′ is a measure of the mass produced during minor growth. The sum of these areas (adjusted for the difference in protein content of creative components of major and minor phases) is a measure of mass produced in the traverse from point 0 to point 9. Using Fig. 10 of the third paper, (p. 256) as a basis, the time to produce this mass is taken as 29.75 years.

The area lying between the male closing path 9-0 and the S-axis is a measure of the mass which would be lost in negative growth in the traverse from point 9 to point 0. If this area were half of that under the combined major and minor growth paths and the traverse time were the same as that from point 0 to point 9, then two passes would be necessary to bring mass to zero at point 0. Similarly a one-third area would require three passes. In other words, traverse time requirements are inversely proportional to the areas.

$$\frac{\text{Time 9 to 0}}{\text{Time 0 to 9}} = \frac{\text{Area under growth paths}}{\text{Area under closing path}}$$

This proportion of areas is found to be 5.1 : 1. Therefore, the time required to make the traverse from point 9 to point 0 (if completion of that traverse were possible) would be

$$5.1 \times 29.75 = 152 \text{ years}$$

and the age at which point 0 would be reached would be

$$29 + 152 = 181 \text{ years}$$

Food capacity achieved in the major phase at point 9 is 6f (3, p. 252). That achieved since origin 0 is 7f (3, p. 260). Along path 9-0, the food capacity-time expression based on Exp. 17 (3, p. 253) becomes

$$(c + s) = \frac{7f}{152}\left( 2t - \frac{t^2}{152} \right)$$

This applies to the male path.

In the case of the female pattern 6.75-17.9, point 7 is reached at age 50 (3, p. 257). The time required to traverse from point 7 to point 0 is

$$181 - 50 = 131 \text{ years}$$

Food capacity achieved in the major phase is 4f; that achieved since origin 0 is 5f. The food capacity-time relationship then becomes

$$(c + s) = \frac{5f}{131}\left(2t - \frac{t^2}{131}\right)$$

With these formulas it now becomes possible to close the cycle as is done in Fig. 2.

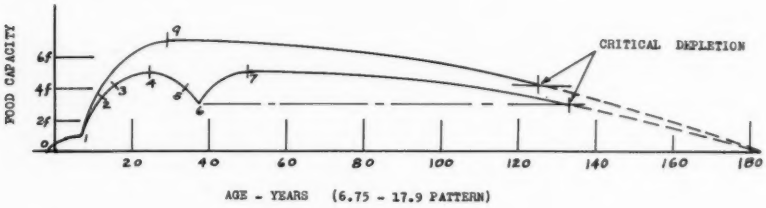


FIGURE 2
Food Capacity and Critical Depletion Based on Cycle Paths.

Decline along the closing paths appears as decrease in both mass and food capacity. Because of the resistance of some portions of the human body—notably the skeleton—to application in negative growth, decrease in mass cannot keep pace with decrease in food capacity. There is a point beyond which the depleted capacity for food is no longer sufficient to keep the body functioning as a living organism and death follows. We will call such a point a point of critical depletion.

In the case of the female, a point of critical depletion already exists at point 6 (paper 3, p. 249). There, with capacity for vital functions depleted to the limit under full reproduction, death would have ensued but for the insertion of the capacity recovery path. It becomes a simple matter, therefore, to locate the point of critical depletion on the female closing path either by computation or by projection as shown.

Since, in the closing phase, the life of the female is no longer complicated by the requirements of reproduction, it seems logical to

assume that the same ratio of food capacity at critical depletion to food capacity at point of maximum applies to female and male alike. In other words, a man's food capacity at point of critical depletion should be

$$\frac{3f}{5f} \times 7f = 4.2f$$

and this level is also indicated on Fig. 2.

Critical depletion for the female of the cycle pattern 6.75-17.9 and for the male of the corresponding pattern is now found to occur at the ages of 133 and 125. Fig. 1 satisfies the mathematical requirement of closing the cycle. Fig. 2 brings the representation of human life to its complete proportion.

Here we have an interpretation of human life completed without impairment by deficiency, over-indulgence or physical misfortune and evolved to the point of highest biological efficiency.

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# SPIRAL GROWTH OF FUNGUS COLONIES

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We have met several times in our laboratory a pattern of mold growth which is so unusual as to merit notice. Instead of growing radially from a point of inoculation, as fungus mycelium ordinarily does, some hyphae grow out with a definite bend, the curvature being toward the left or counterclockwise as seen when one is looking down on the surface of the agar bearing the colony. Four strains of molds have exhibited the phenomenon: an *Aspergillus*, a *Phoma*, and two *Curvulariae*.

The most intensively studied strain was an isolant of *Aspergillus ustus* (Bain.) Thom and Church which was originally obtained by the Chemical Warfare Service of the United States Army in 1943 from a piece of decaying cloth in Milne Bay, New Guinea. The behavior of the mycelium is best seen in a fast-growing culture about a week old, when the colony is 50 to 60 mm. in diameter, as shown in Fig. 2. Hyphae at the extreme edge of the colony do not grow to the left, but push radially out as is usual in agar cultures of molds. In a moist agar, the peripheral hyphae are submerged. See region A of Fig. 1. These pioneering hyphae cannot be seen in the photograph. Just back of the margin, where the hyphae are matted together, only a vague indication of turning is discernible (region B), but about 2 mm. back of that, masses of hyphae are bunched up in points like tiny, wet watercolor brushes. In the most striking instances, they point as much as ninety degrees left of the colony radius, but never more than that (region C). When the tips of the points dry up, aerial hyphae grow in undirected bunches, making the colony surface resemble white caps on wind-blown water (region D), and partially obscure the directional growth of the moist, matted mycelium below. Eventually these non-directional, aerial hyphae completely hide the directed ones, until the older portions of the colony simply appear

## EXPLANATION OF PLATE

Figure 1—Diagrammatic drawing, peripheral mycelium of *Aspergillus ustus,* showing counterclockwise directional growth of hyphae. × 3.

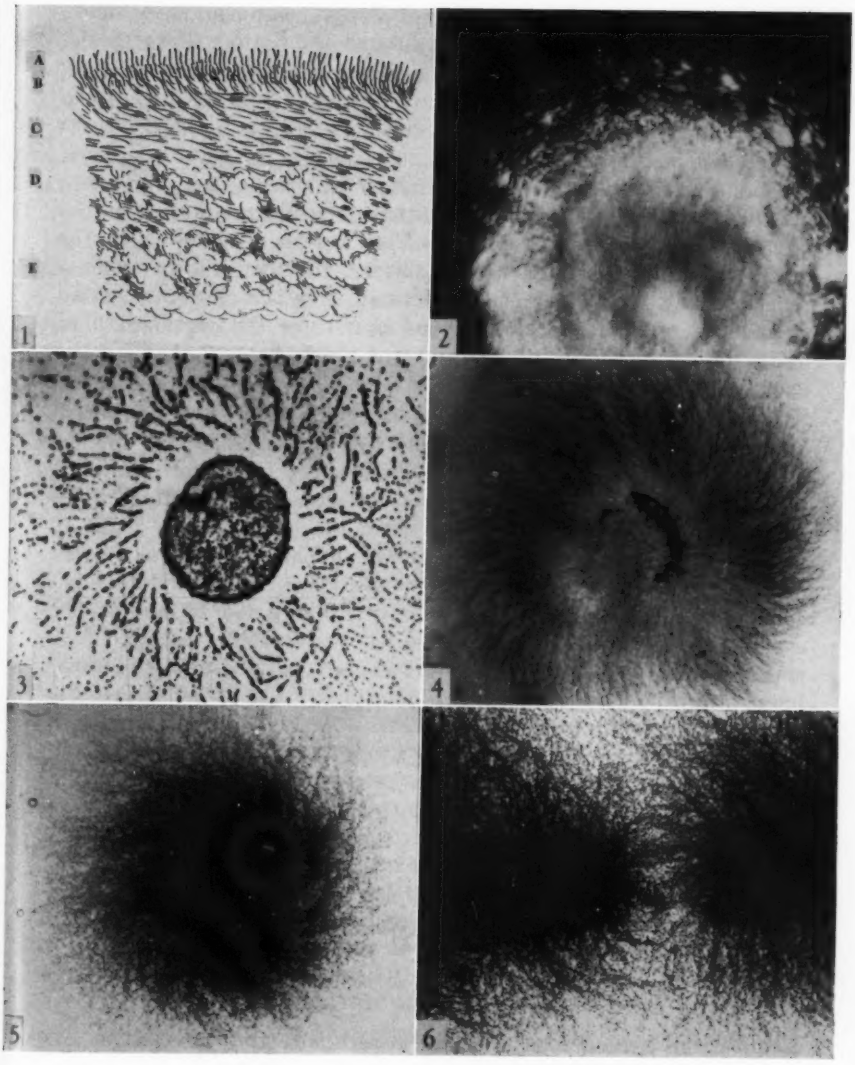
Figure 2—Portion of colony of *A. ustus,* showing extreme degree of twisting. × 1.5.

Figure 3—Colony of *Phoma* sp., showing counterclockwise spiraling lines of pycnidia. × 2.5.

Figure 4—Colony of *Curvularia* sp. The twisting vegetative mycelium is stained green, and was photographed through a red filter. Sporulation in center only. × 2.5.

Figure 5—*Curvularia interseminata,* colony in sporulating phase, with curving lines of dark conidia. × 2.5.

Figure 6—The edges of two juxtaposed colonies of *C. interseminata,* showing influence of mycelia on each other, with some strands of conidium-bearing hyphae turning clockwise. × 2.5.


A
B
C
D
E
1
2
3
4
5
6

fluffy (region E). The spiral growth habit, however, still shows through the agar and can be seen through the bottom of the plate.

The *Phoma* is an undescribed species which was isolated in July, 1960, from decaying wood in salt water near Jones Beach, Long Island, New York. The genus produces its conidia in dark, flask-shaped bodies, the pycnidia, which in most species are scattered at random over the surface of the culture. In this strain, however, many pycnidia are produced in lines which have a tendency to curve to the left. In the dark, central area in Fig. 3, which shows where the inoculating loop touched the agar surface, the pycnidia are not arranged in any pattern. Outside the central area is a ring in which no pycnidia were produced, and beyond that come the organized lines of pycnidia which indicate the outward growing path taken by the main vegetative hyphae.

Both the *Curvulariae* are isolants from sea water. One, *C. interseminata* Berkeley and Ravenel, was collected in Delaware Bay in 1957, and has been cultivated in this laboratory from that time until 1960 without showing any tendency to grow in a spiral pattern. The second, an undescribed species, is from the Jones Beach area. It began to grow with directionally curved hyphae as soon as it was brought into cultivation. In this genus, dark-colored, microscopic conidia are produced in sufficient numbers to be visible to the unaided eye. In most strains, the conidia so nearly cover the colony that it appears rather uniformly grayish-brown, but in these unusual forms, lines of conidiophores, following the main strands of vegetative hyphae, make conspicuous dark streaks. In Fig. 4, the conidia are limited to the dark, central crescent where the inoculating loop made contact. Because the mycelium in the periphery had not yet gone into its reproductive phase, the transparent hyphae were stained to give them contrast for photography. Fig. 5, however was made from an untreated culture, and only the dark, stippled lines of conidia can be seen, although the colorless peripheral mycelium of the original colony could, with proper illumination, be seen spiraling out beyond the conidium-bearing portion.

## Experimental Methods

When the phenomenon of spiral growth was first observed in *Aspergillus,* it occurred when the organism was being cultivated on a

dextrose-peptone agar. When, for diagnostic purposes, the mold was transferred to a sucrose medium, the spiral disappeared and the mycelium grew out in a normal, radial fashion. Transferred back to dextrose, it again grew in a spiral. The spiral growth seemed to depend upon a nutrient factor, but after several generations on sucrose agar, the spiral would return, although it did not do so constantly or dependably. It did, however, appear regularly on the dextrose-peptone medium during about a year of continuous culture.

In an effort to discover the factor responsible for the curvature, a number of experiments of two types were made: nutritional experiments and experiments on the physical environment.

From the results on a variety of nutrient media, the most definite conclusion that can be made is that any set of conditions which favors vigorous growth also enhances the tendency to spiral. The most conspicuous curvature appeared when the fungus was grown on agar containing dextrose, peptone, malt extract, and mineral salts as in Czapek's agar ($NH_4NO_3$, $MgSO_4$, KCl, $FeSO_4$, and $KH_2PO_4$). By omitting one ingredient at a time, one can reduce the general vigor of growth, and thereby lessen the degree of curvature, but *Aspergilli* are not fastidious and will survive on media which would be useless to more demanding organisms. No one of the ingredients, either organic or mineral, is responsible for the spiral pattern.

Temperature does not affect curvature except insofar as it does, at high or low extremes, affect general growth and therefore indirectly the tightness of the spiral. Neither does the acidity or alkalinity of the medium. Culture were grown at pH 3.0 to 8.0 in steps of 0.5. At pH 3.5 to 5.0, curling was maximal. Above or below those points, it was weaker and slower to appear, but it did eventually show up even at pH 8.0, although it required eleven days at that level of alkalinity as contrasted with strong twisting in four days at pH 4.5.

If the direction of growth were influenced by forces in the general environment, such as electrical fields in incubators or the gravitational field of the earth, it would always be constant with respect to the direction of those outside forces. In fact, the mycelium turns, when it turns at all, to the left with respect to the surface of the medium, regardless of the position of the culture dish. Directional growth is unaffected by asymmetry of illumination or heat. Incubating the cultures on clinostats, revolving either clockwise or counterclockwise at 60 r.p.m., made no difference in the manner of growth.

Because the curvature was uniformly unidirectional with respect to the agar surface, two attempts were made to alter the surface. In one, the firmness of the agar was varied by increasing the amount of agar in the medium from 1% to 4% in 1% steps. All colonies twisted, although less strongly on the 1% and 4% than on the 2% and 3% agar media. No spiral growth has ever appeared on liquid media. In another run, after Petri dishes were partially filled with agar, the fungus was inoculated on the surface and allowed to grow until twisting was evident, at which time cool, liquid agar was poured over the colony until the dish was full. Growth stopped.

When the spiral *Aspergillus* was taken from Washington, D. C., in 1946, to the West Virginia University, all curvature ceased, and the cultures grew straight and radial, even when the media were prepared from the same bottles of reagents that had been used in earlier trials. At that time, d-glutamic acid was incorporated into the glucose medium, with the possibility that the unnatural isomer might induce a return to the counterclockwise bending. Under the conditions then prevailing, the addition of that compound made no difference in the growth pattern. Removal to New York had no effect, radial growth continuing. But when the cultures were taken back to Washington in 1948, spirals reappeared, and remained constant under a wide variety of conditions. These facts gave rise to a suspicion that some imperceptible local force was acting, especially when a return to New York was accompanied by a reversion to constantly radial growth for the ensuing three years. In 1951, sub-cultures of the fungus were distributed to a dozen laboratories widely scattered throughout the United States. Only two (one in Michigan and one in North Carolina) reported some visible left-hand curvature. The cultures sent out were all transfers from a colony derived from a single conidium, and were therefore clonal. At the same time, a series of transfers was made from that radial culture, and of 20 colonies, five reverted to the spiral habit, which they retained until the following year, when all the cultures were lost during a move of the entire laboratory.

No further spiral growth of mold colonies appeared until the three marine isolants figured in the present paper showed it. They all have some features in common. They turn counterclockwise on the surface of the solid medium, and their curvature is not affected by position,

temperature, or by any of the ingredients in the media that have been tried. It is affected by the general vigor of the plant.

The *Phoma* does occasionally produce a line of pycnidia turning to the right, but this is rare. No general reversal of the curvature has been achieved. Only by growing two colonies close to one another in the same dish can one make some of the hyphae bend in a right-hand direction. The superficial resemblance of the contiguous mycelia to iron filings in a magnetic field is obvious (Fig. 6). This suggests that some diffusable substance may be effective in determining the direction of the turn, and efforts are being made to isolate and characterize this substance. But even if this should prove successful, merely demonstrating such a material will not explain why it determines a directional curvature with respect to a solid surface, or why spiral growth has been so capricious in the past.

## Discussion

Spiral growth of colonies of microorganisms has been most extensively investigated in bacteria, especially *Bacillus cereus* var. *mycoides*. This variety, of which a number of strains are known, produces filamentous strands of cells which push along an agar surface, curving as they go and making intricately twisted and branched curls. The counterclockwise strains, according to Murray and Elder (1949), outnumber the clockwise strains by three to one. Roberts (1937) reported that when he attached one end of a filament to a firm agar surface and left the other end free in a liquid medium, he obtained "perfect correlation between direction of filament twisting and direction of colonial spirality." Hettenbach and Ludwig (1951), using right- and left-handed strains, induced some of them to reverse by incorporating d- and l-isomers of some compounds (tartaric acid and digitonin) in the growth media, or by growing them on agar which had previously supported a strain of opposite tendency, but the reversals were not permanent. They concluded that the commoner left turning strains (or at least some of them) could be reversed by unnatural isomers, as l-tartaric acid, while the right-handed strains responded to the natural isomers, as d-tartaric acid.

In filamentous fungi, emphasis has generally been on the spiral growth of individual hyphae. Even when producing a straight, erect sporangiophore, a hypha of *Phycomyces* grows in a spiral fashion, but

in such an instance the colony as a whole does not have a spiral appearance. In fact, the manner of growth of the single hypha cannot be observed unless markers are placed on its surface (Castle, 1942). A different kind of twisting growth occurs in the helical threads of some fungi, as in *Epidermophyton* (McCormack and Benham, 1952). *Chaetomium* frequently has perithecial hairs which may be as spiral as watch springs, as in *C. circinatum,* or in helical coils, as in *C. tortile* and *C. spirale.* The helical turns are counterclockwise as seen from apex to base, with only rare exceptions. One species, *C. contortum,* has hairs in which the turns are rather regularly reversed with clockwise and counterclockwise turns alternating (Chivers, 1915).

The only published work I have found on spiral growth in fungus colonies is by Delitsch (1943), who has two photographs of *Penicillium pinophilum* Hedgcock (*P. funiculosum* Thom) which clearly spirals counterclockwise. One, on milk agar, twists slightly, and the other, on milk agar with sodium carbonate, twists strongly. Two other photographs of the same strain, grown on wort agar and on milk agar with 3% NaCl, show typical, radial colonies. No mention of the unusual growth habit appears in the text, although the spirality of the actual colonies must have been striking.

Spirality in bacterial colonies results from extension of filamentous strands of cells which slide along a solid surface, with all the cells of the filament undergoing repeated division and enlargement. The cells at the ends of filaments are thus pushed forward by the extension of the older parts. Some algae, *Ulothrix,* for example, grow similarly, except that in the strains in this laboratory, the spiral is about as often clockwise as counterclockwise. This indicates that the coiling, which is often mechanically regular in form, is a result of a random bending of a filament tip which continues, once it has been started in a given direction, to be pushed on around as a result of intercalary growth along the filament. Inasmuch as filamentous fungi increase in length by apical and not by intercalary extension, a spiral pattern in the fungus colony is produced in a manner somewhat different from that of a bacterial or algal colony.

In spite of the differences, bacterial and fungal spirals have some features in common. In bacteria, the growth is predominantly counterclockwise, or to the left, although some clockwise strains, both natural and induced, have been reported. In all fungi met so

far, any non-radial growth has been counterclockwise. Another point of resemblance is the curvature, in all instances, in a persistent direction regardless of the position of the agar surface (Murray and Elder, 1949). Finally, all spiraling cultures exhibit a baffling genetic behavior, in which direction is not dependably constant. The inconstancy has been less marked in the bacteria than in the fungi, in which strains have grown in one form for years, then changed without any obvious change in treatment, but without doing so in a dependable fashion. The nearest approach to control of the phenomenon was made by Hettenbach and Ludwig on *B. mycoides,* but even they succeeded in obtaining only partial reversals. One cannot inoculate a plate and predict confidently the character of the forthcoming colony.

At present, we know that spiral growth (1) can be inherited, although whether the control is through nuclear genes is not known, and (2) is mediated by extrinsic factors, some being purely physical (a surface effect), and some nutritional, as was shown in our dextrose-to-sucrose transfers and in the isomer experiments of Hettenbach and Ludwig.

I wish to thank Misses Bette Ruth Goldstein and Mary Ann Steponanko who, under a grant from the National Science Foundation to Yeshiva University, helped with culture work and photography.

## Summary

A counterclockwise spiral pattern of growth in four strains of fungi is reported. The curvature is not constantly present, but is expressed or repressed by unknown genetic and environmental factors. Experimental attempts to control it included variations in nutrition, pH, light, temperature, geographic location, movement and position of the colonies. Spiral growth in fungi is compared with that in other microorganisms.

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# ESTROGEN-INDUCED SERUM PROTEIN SYNTHESIS IN THE LIVER OF THE CHICKEN EMBRYO*

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Since serum proteins are synthesized mainly in the liver, their concentrations in the serum can, under certain circumstances, be taken as reflections of the synthetic capability of the organ. Thus, in the case of the chicken embryo, physiological differentiation of the embryonic liver cells can be followed broadly by noting concentrations of the various serum proteins at different stages of development. In addition, the absence or presence of individual types of serum proteins may provide significant insights into the status of the liver cells at any given time.

As an example of the type of data and the implications that may be forthcoming from such studies, the case of chicken embryos of 7-10 days of incubation may be considered. In the serums of these less differentiated forms, light lopoproteins ($s_f$4-10) are present in higher concentration than in more advanced stages; albumins, lipoproteins of $s_f$10-20, denser lipoproteins (-s3-6) and mucoproteins are present in very much lower concentration; and lipoproteins of $s_f$0-4 and -s6-10 cannot be detected (1-3). These data suggest that the rapidly dividing cells of the young embryo are relatively poorly geared to the synthesis of cholesterol, phospholipid, triglyceride, polysaccharides, mucoproteins and even pure serum polypeptides such as albumin. The alternative, that these syntheses can take place efficiently in these cells but that the "directive" is lacking, can only be tested by supplying a "directive."

With the discovery that administration of estrogens results in the elicitation of three unique serum proteins by the liver of the adult

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fowl (4),[1] the capacity of the embryonic liver to synthesize and secrete several of the above named serum components could be investigated. From another perspective, it could be determined (a) whether the liver, even in relatively early stages of development, is capable of responding to estrogens and if not, (b) the exact stage wherein competence is present for synthesis of each of the three proteins mentioned. It could, with a fair degree of certainty, also be determined (c) if the syntheses of all three proteins are inseparably linked with one another or with other known metabolic events taking place during the development of the liver.

## Materials and Methods

For the present studies, embryonated eggs of 9, 10, 15 and 18 days of incubation and newly hatched chicks (21 days of incubation) were injected with 5 mg. of estrogenic substances (Ayerst Co.) each and were bled 3, 4 or 6 days following such injection. Saline injected eggs and chicks served as controls. Pooled serums from each of the above groups were assayed for (a) content of calcium (Table I) and (b) content of light and dense lipoproteins, $X_1$-phosphoprotein and $X_2$-lipoglycoprotein, as well as albumin and denser globulins. The amount of serum calcium in excess of the normal value (10 mg. %) was taken as a reflection of the amount of $X_1$-phosphoprotein present in the serum, since it has been shown that in estrogenized birds the serum calcium above this value is largely (>90 per cent) bound to phosphoprotein (4,5). Analyses of lipoproteins, $X_2$-lipoglycoprotein, albumin and dense globulins were carried out with the aid of the ultracentrifuge (Figure 1). Total lipids of sera were determined by means of the extraction procedures of Delsal (6) (Table II) and

[1] Serum proteins elicited by estrogen treatment include serum $X_1$-phosphoprotein, serum $X_2$-lipoglycoprotein and a lighter lipoprotein (4-5). In the chick these are characterized by svedberg consants S8.5, S16.5 and $S_f$10-100, respectively. The phosphoprotein has been shown to carry most of the elevated calcium in the serums of these fowl. The lipoglycoprotein contains approximately 20 per cent lipid, mainly phospholipid, and the lipid moiety of the lighter lipoprotein (80% of the total molecule) consists mainly of sterol ester and phospholipid with varying amounts of triglyceride. Except in the laying hen, these proteins are rarely present in the serum in significant concentration. Flickinger and Rounds (Biochim. et Biophys. Acta 22, 38 (1956)) used $P^{32}$ to trace the course of rapidly synthesized phosphoproteins in laying hens. These were found to originate in the liver of the hen, appeared briefly in the blood and finally were deposited in the yolks of the eggs.

constituent lipids were ascertained by silicic acid chromatography (7) (Table III).

## Results

As illustrated by ultracentrifuge patterns of whole serums in Figure 1 and by data on serum lipids presented in Tables II and III, estrogen treatment was followed by increases in lighter serum lipoproteins in all stages of embryos challenged.[2] The lipid responses were, however, graded in intensity, the youngest embryos showing the smallest effect and hatching chicks (injected at 15 or 18 days of incubation) displaying the largest increases.

None of the embryos injected revealed any detectable increase in

TABLE I
Total Serum Calciums

| Number of Animals | Age Estrogen Given (day of incubation) | Age Bled (day of incubation) | Food | Calcium (mg. %) |
|---|---|---|---|---|
| 24 | 9th day | 13th day | Yolk sac | 11.8** |
| 24 | 10th day | 14th day | Yolk sac | 14.0 |
| 24 | Control (no estrogen) | 13th day | Yolk sac | 10.4 |
| 24 | Control (no estrogen) | 14th day | Yolk sac | 12.4 |
| 15 | 15th day | 18th day | Yolk sac | 13.0 |
| 15 | 15th day | 18th day | Yolk sac | 8.9 |
| 15 | Control (no estrogen) | 18th day | Yolk sac | 12.2 |
| 15 | Control (no estrogen) | 18th day | Yolk sac | 9.1 |
| 12 | 15th day | 21st day | Yolk sac | 14.4 |
| 12 | 15th day | 21st day* | Yolk sac | 13.7 |
| 12 | 18th day | 21st day* | Yolk sac | 15.8 |
| 12 | Control (no estrogen) | 21st day* | Yolk sac | 16.8 |
| 12 | Control (no estrogen) | 21st day* | Yolk sac | 9.9 |
| 12 | Control (no estrogen) | 21st day* | Yolk sac | 10.9 |
| 12 | 21st day | 24th day*** | Chick mash | 31.7 |
| 12 | 21st day | 24th day*** | Chick mash | 27.3 |
| 12 | 21st day and 23rd day | 24th day*** | Chick mash | 26.5 |
| 12 | Control (no estrogen) | 24th day*** | Chick mash | 14.0 |
| 12 | Control (no estrogen) | 24th day*** | Chick mash | 13.6 |
| 12 | Control (no estrogen) | 24th day*** | Chick mash | 11.2 |

* day of hatching
** all calcium values given are the means of duplicate analyses
*** 3-day-old chick
Note that calcium-binding (by phosphoprotein) occurs only in the groups injected with estrogen on the day of hatching.

[2] In Table III the composition of the total serum lipids of each age group treated with estrogen is shown to be different from that of the controls chiefly by virtue of a decrease in sterol ester and an increase in triglyceride. This attests to the qualitative similarity of the light lipoprotein response in different age groups following estrogen treatment and further indicates that the mechanisms of synthesis of this material are similar in each age group.

serum $X_1$-phosphoprotein (Fig. 1, Table I) or serum $X_2$-lipoglycoproteins. Increases of these proteins were observed only when the estrogen was administered post-hatching (Fig. 1, Table I).

Accompanying the increase in lighter lipoproteins, was a decrease in the amount of serum albumin. There was also a slight decrease in dense lipoprotein (Figure 1) in 18 days and older animals. Adult birds, responding more strongly to estrogen treatment in terms of production of $X_1$-phosphoproteins and $X_2$-lipoglycoprotein, always show a striking decrease in concentration of this protein (8) in the serum.

TABLE II
TOTAL SERUM LIPIDS

| Number of Animals | Age Estrogen Given (day of incubation) | Age Bled (day of incubation) | Food | Lipid (mgs/ml) |
|---|---|---|---|---|
| 24 | 9th day | 13th day | Yolk sac | 21.6-21.0 |
| 24 | 10th day | 14th day | Yolk sac | 26.0 |
| 24 | Control (no estrogen) | 13th day | Yolk sac | 13.2-13.4 |
| 24 | Control (no estrogen) | 14th day | Yolk sac | 16.6 |
| 15 | 15th day | 18th day | Yolk sac | 37.4 |
| 15 | 15th day | 18th day | Yolk sac | 31.0 |
| 15 | Control (no estrogen) | 18th day | Yolk sac | 19.4-19.8 |
| 15 | Control (no estrogen) | 18th day | Yolk sac | 16.8 |
| 12 | 15th day | 21st day* | Yolk sac | 58.2-58.4 |
| 12 | 15th day | 21st day* | Yolk sac | 36 6-38.0 |
| 12 | 18th day | 21st day* | Yolk sac | 49.0-48.0 |
| 12 | Control (no estrogen) | 21st day* | Yolk sac | 17.2 |
| 12 | Control (no estrogen) | 21st day* | Yolk sac | 16.4-16.6 |
| 12 | Control (no estrogen) | 21st day* | Yolk sac | 14.8 |
| 12 | 21st day | 24th day** | Chick Mash | 36.0-35.8 |
| 12 | 21st day | 24th day** | Chick Mash | 27.4-27.4 |
| 12 | 21st day | 24th day** | Chick Mash | 32.0 |
| 12 | Control (no estrogen) | 24th day** | Chick Mash | 20.4-20.4 |
| 12 | Control (no estrogen) | 24th day** | Chick Mash | 20.1 |
| 12 | Control (no estrogen) | 24th day** | Chick Mash | 20.0 |

* day of hatching
** 3 days post-hatching

## DISCUSSION

In terms of serum light lipoprotein ($S_f$10-100, Figure 1), a significant response was seen in all embryos tested. Thus it is certain that estrogens, injected into the yolk sac, in some manner attain their intended objectives, namely the livers of the embryos.

The increasing yield of light lipoprotein up to hatching was probably due to an increasing capacity of the liver to respond in this

TABLE III

CONSTITUENT SERUM LIPIDS OF CONTROL AND ESTROGENIZED EMBRYOS AND CHICKS

| Number of Animals | Age & Treatment | Sterol Ester % | Triglyceride % | Sterol % | Phospholipid % |
|---|---|---|---|---|---|
| 24 | 13th day (Estrogen) | 12.8 (12.5-13.0) | 34.1 (27.9-40.2) | 7.7 (6.7-8.7) | 45.5 (38.0-52.9) |
| 24 | 13th day (Control) | 24.3 (23.2-25.4) | 24.0 (19.0-29.0) | 4.5 (3.2-5.8) | 47.2 (42.0-52.4) |
| 15 | 18th day (Estrogen) | 26.6 | 33.3 | 5.7 | 34.4 |
| 15 | 18th day (Control) | 23.3 | 24.4 | 4.4 | 47.8 |
| 24 | 21st day* (Estrogen) | 22.6 (21.5-23.7) | 42.1 (41.4-42.8) | 3.4 (3.2-3.5) | 31.9 (30.2-33.6) |
| 12 | 21st day (Control) | 39.5 | 11.6 | 4.6 | 44.2 |
| 24 | 24th day** (Estrogen) | 27.4 (26.0-28.7) | 25.6 (24.7-26.5) | 4.2 (4.0-4.4) | 42.8 (42.5-43.1) |
| 24 | 24th day (Control) | 42.9 (41.6-44.2) | 8.8 ( 8.0-9.7) | 7.1 (7.1) | 41.2 (40.7-41.6) |

* day of hatching

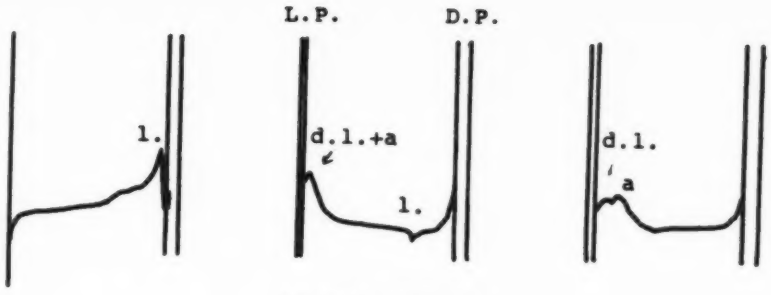
** 3-day chicks

Note that sterol esters are reduced and triglycerides are increased in all estrogenized animals with the exception of the 18-day age group which shows no decrease in the sterol ester fraction but shows similar changes in proportions of triglyceride and phospholipid.

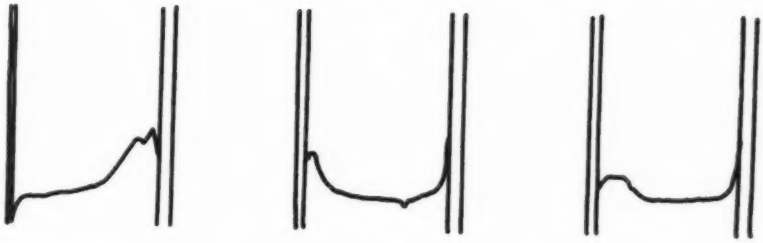
FIGURE 1

Typical ultracentrifuge patterns of serum samples from chicken embryos and chicks with and without injection of 5 mg. of estrogenic substances 3-4 days previously. From left to right pictures were taken 0, 32 and 64 minutes after reaching speed (50740 rpm). Serums from 13-day and 18-day embryos were undiluted but were adjusted to a density of 1.063 by the addition of 8.8 per cent NaCl. Serums from 21-day and 24-day chicks were diluted with one part 17.6 per cent NaCl solution. Patterns in the pictures to the far left have been turned upside down so that the peak remains within the frame. Legend: a = albumin, d.l = dense lipoprotein, l. = light lipoprotein, $X_1$ = $X_1$-phosphoproteins, $X_2$ = $X_2$-lipoglycoprotein, L.P. = pile-up of lipoprotein, d.p. = pile-up of dense protein.

Note (in frames to the far left) that injections of estrogen resulted in increases in light lipoprotein in all cases presented and (in middle and frames to the right) that albumin was decreased at each stage following estrogen treatment. Only in the 24-day chick (injected on the 21st day) was estrogen administration followed by resolvable $X_1$ and $X_2$ components (middle frame, bottom).

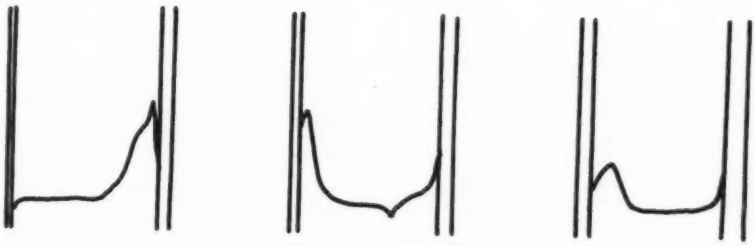



13 DAY CONTROL



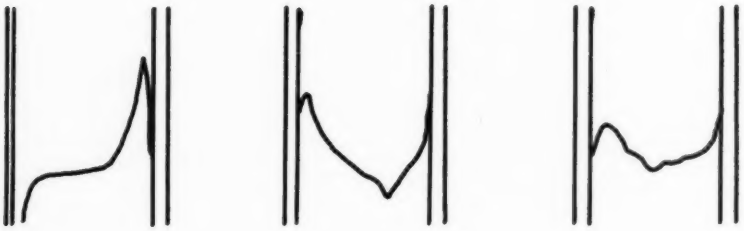
13 DAY ESTROGEN

18 DAY CONTROL

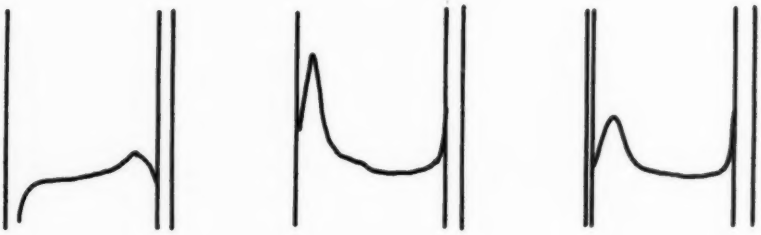


18 DAY ESTROGEN

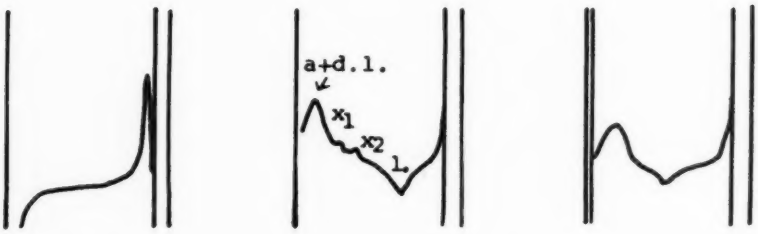
21 DAY CONTROL (dil 1:1)



21 DAY ESTROGEN (dil 1:1)



24 DAY CONTROL (dil 1:1)




24 DAY ESTROGEN (dil 1:1)

fashion to the estrogen stimulus.[3] In any event, the embryonic liver appeared to be a less efficient producer of one or more of the elements required for elicitation of light lipoprotein. The decrease in light lipoprotein observed in chicks sacrificed at 24 days (from start of incubation) was probably due to competition from those systems involved in synthesis of $X_1$-phosphoprotein and $X_2$-lipoglycoprotein. Observed decreases in serum albumin were also probably due to competition with the estrogen-induced proteins. When competition becomes even more pronounced, as in chicks that are synthesizing light lipoprotein, $X_1$-phosphoprotein and $X_2$-lipoglycoprotein all at once, the dense lipoproteins also apparently suffer the effects of this drain on the liver cell's available reserves.

Despite evidence that estrogens were acting (either directly or indirectly) on the liver, synthesis of both $X_1$-phosphoprotein and $X_2$-lipoglycoprotein failed to occur in embryos whereas it took place in the newly hatched chick. The separation in time of light lipoprotein synthesis from that of "$X_1$" and "$X_2$" suggests that the synthesis of the former is independent of the synthesis of the latter two and is possibly primarily related to a shift in the direction of lipid synthesis by the liver, especially of triglyceride and phospholipid. The synthesis of $X_1$-prosphoprotein and $X_2$-lipoglycoprotein do, on the other hand, seem to be related, because one protein has not been detected in the absence of the other.[4]

The age at which competence is achieved with respect to elicitation of $X_1$-phosphoprotein and $X_2$-lipoglycoprotein in the New Hampshire chicken embryo, appears to be approximately 19-20 days of incubation; embryos injected at 18 days of incubation will show $X_1$ phosphoprotein (elevated calcium) in the serum by the day after hatching; a serum protein response should be detectable on the day of hatching if the estrogen were effective on the 18th day of incubation.

It is possible that the embryonic chicken liver is, *per se*, capable of

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[3] Such capacity could be due to the presence of more enzymes, more available substrates, more sources of energy or less competition from syntheses connected with cellular differentiation or division.

[4] Furthermore, (a) these molecules occur together in insoluble form in the yolk granules and (b) $X_1$-phosphoprotein and $X_2$-lipoglycoprotein precipitate each other with antibody-like selectivity when the calcium is removed from the phosphoprotein moiety (4).

producing $X_1$-phosphoprotein and $X_2$-lipoglycoprotein but that (a) the elicitation of these particular proteins is more complex than has been supposed in terms of stimuli involved and that (b) the embryo is lacking in some respect with regard to such a stimulus. Although, the injection of other hormones in conjunction with estrogens may further elucidate this possibility, the evidence that several of the other serum proteins are present in relatively low concentration in young embryos (including estrogen-induced light lipoprotein) would seem to indicate that low capacities for such syntheses actually exist in the rapidly growing and undifferentiated liver.

## Summary

Injection of estrogen into embryonated eggs resulted in the production of only one of three serum proteins that characteristically result from estrogen treatment of the adult fowl and this in relatively low quantity in the younger embryos. The protein elicited was light lipoprotein ($S_f$10-100). $X_1$-Phosphoprotein (S8.5) and $X_2$-lipoglycoprotein (S16.5) appeared in the serum for the first time when estrogen was injected into the yolk sacs of newly hatched chicks. It is tentatively concluded (1) that the mechanisms for synthesis of estrogen-induced light lipoprotein are present in the embryonic liver but are relatively poorly developed in the younger embryos, (2) that the mechanisms for synthesis of $X_1$-phosphoprotein and $X_2$-lipoglycoproteins are absent and cannot be induced, (3) that the synthesis of light lipoprotein is not directly linked to the synthesis of the other two estrogen induced serum proteins but that (4) the synthesis of $X_1$ phosphoprotein and $X_2$-lipoglycoprotein are inseparable events.

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